OXIDATIVE STRESS IN DIABETES AND METABOLIC SYNDROME

SUMMARY

OF THE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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BY

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Metabolic syndrome, which occurs commonly in populations around the world, has been associated with an increased risk for developing cardiovascular disease and diabetes. Typically, measures of excess weight, elevated blood pressure, dyslipidemia, hyperglycemia, and inflammation are considered key elements of the syndrome. In addition to these abnormalities, oxidative stress, which is an imbalance between prooxidants and antioxidants, has also been implicated in the pathogenesis of cardiovascular disease and diabetes and serves as a common feature of metabolic syndrome. This has at least two possible implications. First, oxidative stress may be involved in the pathogenesis of metabolic syndrome. Second, oxidative stress may contribute to the complications that arise from the syndrome. Thus, understanding factors that serve to maintain the balance between prooxidants and antioxidants may help to increase the understanding about its pathophysiology and to define possible avenues for prevention and treatment.

The etiology of metabolic syndrome is multi-factorial and the reports of National Cholesterol Education Program Adult Treatment Panel III (NCEP/ATP III) recognized the importance of cardiovascular risk factors of what they referred to as a "constellation of lipid and non-lipid risk factors of metabolic origin" and designated this cluster as metabolic syndrome. The allied lipid disorder consists of elevations of serum triglycerides, apolipoprotein B (apo B) and small LDL particles, and low levels of HDL. These multiple abnormalities almost certainly promote the development of atherosclerosis. All of the steps in the progression of atherosclerosis in one way or another are inflammatory and it is well documented that inflammatory mediators (such as CRP and TNF-α) play a paramount role in the initiation, progression and rupture of atherosclerotic plaques. Thus markers of inflammation and endothelial dysfunction may provide additional information about a patient's risk of developing metabolic syndrome and may become new targets for treatment.

On the other hand, evidence has emerged suggesting that the free radical generation or inadequate antioxidant defenses may lead to alteration in

structure and function of fat, protein, carbohydrate, RNA or DNA molecule. As far as the management of these disturbances in the body itself is concerned, an HDL-associated enzyme paraoxonase (PON-1) has emerged as one of the best prototypes in context of metabolic syndrome. In this regard, an understanding to the mechanism and potential role played by antioxidants, gained an outstanding importance. This has further stimulated intense research efforts to validate the beneficial effects of exogenous antioxidants or certain medicinal interventions against diabetes, hypertension and metabolic syndrome.

The first chapter of the thesis highlights and reaffirms the involvement of abnormal lipid profile together with the elevated waist circumference and inflammatory markers in prediction of metabolic syndrome in diabetic and hypertensive population. It has been established that CRP levels >3.0 mg/l adds a prognostic evidence for metabolic syndrome. Subsequently, our findings demonstrating such higher values of CRP put both diabetic and hypertensive subjects at a higher risk to develop metabolic syndrome. However, the comparatively lowered PON-1 activity and significantly lesser time for oxidation of sdLDL among hypertensive subjects reflected them to be a much easier prey. This adds to a novel finding in the field of metabolic syndrome suggesting and giving an idea to find urgent preventive measures to control hypertension as well as diabetes.

The second chapter of the thesis therefore accounts for the strategies involved in the treatment of diabetes and hypertension. To explain the depth of its significance, this chapter has been bifurcated in two divisions. The treatment modalities for diabetic subjects included monotherapy with either metformin or insulin. Our aim was to scrutinize all the traits of metabolic syndrome before and after the supplementation of these hypoglycemics with the recommended doses for a considerable period of time. It was noticed that despite of beneficial changes in lipoprotein profile, such a monotherapy was not able to recompense for increased inflammatory markers and the DNA damage incurred in diabetic population. The second unit to this chapter throws light on the efficacy of

antihypertensive drugs over the various abnormal parameters of metabolic syndrome. Our results have demonstrated a significant elevation in PON-1 activity together with a reduction in lipid peroxidation as well as levels of inflammatory markers in hypertensive subjects treated with either ramipril or losartan as a once-daily anti-hypertensive agent. Besides, it was also witnessed that none of the two therapies could prevent the DNA damage up to any conspicuous level.

If oxidative stress is the pathogenic mechanism leading to metabolic syndrome as well as diabetes, the ability of a drug to prevent or reverse oxidant stress can account for its clinical usefulness. In view of this, the third chapter discusses the role of antioxidants, particularly vitamin C and vitamin E in prevention of the metabolic abnormalities aroused in the alloxan-induced diabetic rabbits. Our results with vitamin C and E clearly demonstrated significant improvement in each of the component of metabolic syndrome, the former showing much favorable results however. In addition, the DNA damage was also recovered partially, when they were supplemented with vitamin C. Such a favorable effect with antioxidants may be attributed to their ability to maintain the balance between excess free radicals and reduced antioxidant defenses.

Hitherto, it has been recognized that metabolic syndrome represents a very heterogeneous cluster of components that need a tailored but integrated approach. Moreover, the challenge for developing a new drug that will substantially reduce multiple risk factors is formidable. In this direction, the present study successfully highlights the importance of antioxidants in ameliorating the complications of metabolic syndrome. It may thus be implicated that antioxidants might provide a better prevention to metabolic syndrome as compared to that conferred by hypoglycemics or antihypertensives. This may also suggest a strong association between diabetes, oxidative stress and metabolic syndrome.

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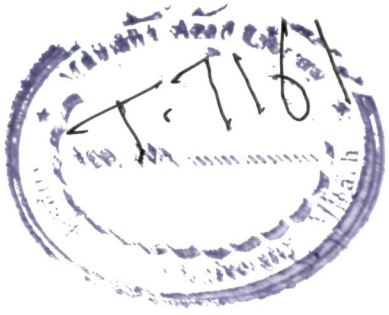

Dedicated to

My Loving Parents

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Dated: 17th Nov 08.

Certificate

This is to certify that the work presented in this thesis has been carried out by **Ms. Nishtha Jain** under my supervision. It is original in nature and has not been submitted for any other degree.

Imrana Naseem
(Reader)

Acknowledgement

Easy it is to tell a fact, but hard it is to know and act!

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Thanks are due to all those people who are directly or indirectly linked to this work. I dedicate this work to my loving parents!

Nishtha 17.11.08

Nishtha Jain

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
Apo-B	Apolipoprotein-B
ARA	Angiotensin receptor antagonist
ARB	Angiotensin receptor blocker
BP	Blood pressure
BSA	*Bovine serum albumin*
$CaCl_2$	Calcium chloride
CRP	C-reactive protein
$CuCl_2$	Cupric chloride
CVD	Cardiovascular disease
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGIR	European Group for the study of Insulin Resistance
ELISA	Enzyme linked immunosorbent assay
FBG	Fasting blood glucose
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HDL	High density lipoprotein
IDL	Intermediate density lipoprotein
IGT	Impaired glucose tolerance
IL-1	Interleukin-1
IL-6	Interleukin-6
LDL	Low density lipoprotein

LMPA	Low melting point agarose
MDA	Malondialdehyde
Mg	Magnesium
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NaOH	Sodium hydroxide
NCEP/ATP III	National Cholesterol Education Programme Adult Treatment Panel III
NPH	Neutral Protamine Hagedorn (insulin)
PBS	Phosphate buffer saline
PON-1	Paraoxonase-1
ROS	Reactive oxygen species
sdLDL	Small dense LDL
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TC	Total cholesterol
TGs	Triglycerides
TNF-α	Tumor necrosis factor-α
VLDL	Very low density lipoprotein
WHO	World Health Organization

Summary

Metabolic syndrome, which occurs commonly in populations around the world, has been associated with an increased risk for developing cardiovascular disease and diabetes. Typically, measures of excess weight, elevated blood pressure, dyslipidemia, hyperglycemia, and inflammation are considered key elements of the syndrome. In addition to these abnormalities, oxidative stress, which is an imbalance between prooxidants and antioxidants, has also been implicated in the pathogenesis of cardiovascular disease and diabetes and serves as a common feature of metabolic syndrome. This has at least two possible implications. First, oxidative stress may be involved in the pathogenesis of metabolic syndrome. Second, oxidative stress may contribute to the complications that arise from the syndrome. Thus, understanding factors that serve to maintain the balance between prooxidants and antioxidants may help to increase the understanding about its pathophysiology and to define possible avenues for prevention and treatment.

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The second chapter of the thesis therefore accounts for the strategies involved in the treatment of diabetes and hypertension. To explain the depth of its significance, this chapter has been bifurcated in two divisions. The treatment modalities for diabetic subjects included monotherapy with either metformin or insulin. Our aim was to scrutinize all the traits of metabolic syndrome before and after the supplementation of these hypoglycemics with the recommended doses for a considerable period of time. It was noticed that despite of beneficial changes in lipoprotein profile, such a monotherapy was not able to recompense for increased inflammatory markers and the DNA damage incurred in diabetic population. The second unit to this chapter throws light on the efficacy of

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Hitherto, it has been recognized that metabolic syndrome represents a very heterogeneous cluster of components that need a tailored but integrated approach. Moreover, the challenge for developing a new drug that will substantially reduce multiple risk factors is formidable. In this direction, the present study successfully highlights the importance of antioxidants in ameliorating the complications of metabolic syndrome. It may thus be implicated that antioxidants might provide a better prevention to metabolic syndrome as compared to that conferred by hypoglycemics or antihypertensives. This may also suggest a strong association between diabetes, oxidative stress and metabolic syndrome.

Literature Review

METABOLIC SYNDROME

The metabolic syndrome is conceptualized as a constellation of metabolic and anthropometric abnormalities (Meigs, 2002), which include hyperglycemia, hypertension, dyslipidemia, central obesity, and excess body weight. Measures of insulin resistance, inflammation, thrombosis, hyperuricemia, and renal function have also been considered for inclusion. The triad of hyperglycemia, hypertension, and hyperuricemia was described as early as 1923 (Kylin, 1923). A major milestone in the history of this syndrome occurred in 1988, when Reaven proposed the concept of syndrome X, which he described as the co-occurrence of resistance to insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, increased very low-density lipoprotein triglyceride, decreased high-density lipoprotein (HDL) cholesterol, and hypertension (Reaven, 1988). This study stimulated renewed interest in the syndrome.

In 1998, the World Health Organization (WHO) proposed a formal definition of the metabolic syndrome (Alberti and Zimmet, 1998). Three years later, the National Cholesterol Education Programme Adult Treatment Panel III (NCEP/ATP III) proposed its definition of the metabolic syndrome (National Institutes of Health, 2001). The European Group for the study of Insulin Resistance (EGIR) also developed a definition (Balkau et al., 2002). The attention that the NCEP/ATP III report brought to the metabolic syndrome has ignited an intense interest, as evidenced by the numerous publications and meetings concerning the metabolic syndrome. Efforts by WHO, NCEP/ATP III, and EGIR to develop standard definitions have been critical in trying to determine the prevalence of this syndrome. Although they share similarities, they also differ considerably.

To meet the WHO definition, a person must have glucose intolerance or insulin resistance plus two of the following four criteria: central obesity, hypertension, dyslipidemia, and albuminuria. In 1999, a modification to the WHO definition was proposed: the blood pressure threshold was lowered from

160/90 mm Hg to 140/90 mm Hg, and the albumin: creatinine ratio threshold was raised from 20 mg/g to 30 mg/g (World Health Organization, 1999). To meet the NCEP/ATP III definition, a person must have three of the following five abnormalities: abdominal adiposity, hypertension, hypertriglyceridemia, low HDL, or hyperglycemia. Of note is that WHO and NCEP/ATP III included diabetes in their definitions, but there has been extensive debate about the appropriateness of including persons with diabetes in prevalence estimates of metabolic syndrome. The EGIR definition specifically excludes diabetes and to meet the definition, a person must have two of the following four criteria: abdominal obesity, hypertriglyceridemia, hypertension, and insulin resistance. Although all three definitions include a measure of abdominal obesity, elevated blood pressure, dyslipidemia, and hyperglycemia, the exact measures and cut points used to define elevations differ among the three definitions as represented in the Table I (TI).

RISK FACTORS FOR METABOLIC SYNDROME

The metabolic syndrome is comprised of a clustering of metabolic risk factors in one individual. The syndrome has been identified as a multidimensional risk factor for cardiovascular diseases (CVD). It is also associated with an increased risk for type 2 diabetes, which in turn is a major risk factor for CVD. The metabolic risk factors for CVD that make up the metabolic syndrome do not directly cause type 2 diabetes but are frequently associated with it. Specifically, following five criteria are listed as the major metabolic risk factors for metabolic syndrome: Atherogenic dyslipidemia {that comprises of elevated apolipoprotein B (apo-B), including elevated serum triglycerides (TGs) plus small low density lipoprotein (LDL) particles and low levels of HDL}, elevated blood pressure, elevated plasma glucose, prothrombic state, and finally the proinflammatory state.

Criteria	NCEP/ATP III	World Health organization	European Group for the study of Insulin Resistance
	≥ Three of the following five criteria:	Presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance plus ≥ two of the following four criteria:	No diabetes plus ≥ two of the following four criteria:
Central Obesity	1. Waist circumference >102 cm in men and >88 cm in women	1. Waist to hip ratio of >0.9 in men or >0.85 in women and/or body mass index >30 kg/m^2	1. Waist circumference ≥ 94 cm for men, ≥ 80 cm for women
Dyslipidemia	2. Triglycerides ≥150 mg/dl (1.695 mmol/l)	2. Triglycerides ≥150 mg/dl (1.695 nmol/l) and/or high density lipoprotein cholesterol <35 mg/dl (0.9 mmol/l) in men and <39 mg/dl (1 mmol/l) in women	2. Triglycerides ≥190 mg/dl (2mmol/l) or high density lipoprotein cholesterol concentration <40 mg/dl (1 mmol/l) or treatment
	3. Low high density lipoprotein cholesterol: < 40 mg/dl (1.036 mmol/l) in men and <50 mg/dl (1.295 mmol/l) in women		
High blood pressure	4. Blood pressure ≥130/85 mm Hg.	3. Blood pressure ≥160/90 mm Hg[a]	3. Blood pressure: systolic blood pressure ≥140 or diastolic blood pressure ≥90 or treatment for hypertension
High glucose	5. Fasting glucose: ≥110mg/dl (≥6.1 mmol/l)		
Other		4. Microalbuminuria: urinary albumin excretion rate ≥20 mg/min or albumin /creatinine ratio ≥20 mg/g[a]	4. Insulin resistance or fasting insulin concentration above the upper quartile for non-diabetic subjects

[a] In 1999, revised WHO criteria were published. Elevated blood pressure was defined as ≥140/90 mm Hg and the albumin: creatinine ratio was increased to 30 mg/g.

TI: Definitions of the metabolic syndrome

Atherogenic dyslipidemia

This lipid disorder consists of elevations of serum TGs, apo-B and small (LDL) particles, and low levels of HDL. It can be differentiated from elevated LDL cholesterol, which is the major risk factor for CVD. Many controlled clinical trials show that LDL-lowering therapy reduces risk for CVD (National Cholesterol Education Program, 2002). The connection between atherogenic dyslipidemia and CVD risk is more complicated than for LDL cholesterol; the multiple lipid abnormalities of atherogenic dyslipidemia make it difficult to dissect the contributions of each abnormality to CVD. These multiple abnormalities almost certainly promote the development of atherosclerosis. An important point to make, however, is that elevated total apo-B overlaps with LDL cholesterol. In normal persons, apo-B is mainly carried in LDL, whereas only small amounts are present in very low density lipoproteins (VLDL). When triglycerides are elevated, however, a somewhat greater portion of apo-B is found in VLDL. With atherogenic dyslipidemia, the LDL cholesterol level in the LDL fraction underestimates the number of LDL particles present, because these particles are partially depleted of cholesterol. In atherogenic dyslipidemia, the total apo-B level frequently is abnormally elevated. There is growing evidence that all apo-B-containing lipoproteins are atherogenic. Whether the different types of lipoproteins that carry apo-B (such as VLDL, large LDL, and small LDL) have the same or different atherogenic potential is uncertain. Suggestive evidence points to small LDL particles being particularly atherogenic, but the evidence is not unequivocal. Some of the apparently higher atherogenicity of small LDL may be related to an increased number of LDL particles in the LDL fraction.

Another important component of atherogenic dyslipidemia is a low level of HDL cholesterol. This reduced level may raise the risk for CVD. At least three possibilities exist (Vega and Grundy, 1996). First, HDL may protect directly against the development of atherosclerosis. Second, a low HDL level may indicate the increase in atherogenic apo-B-containing

lipoproteins. Third, low HDL is commonly associated with the nonlipid risk factors of metabolic syndrome and hence is a powerful marker for risk (National Cholesterol Education Program, 2002). A low HDL may also be directly atherogenic because of a deficit in the protective effect of HDL.

Elevated blood pressure

An elevation of blood pressure is one of the components of metabolic syndrome (Grundy et al., 2004). How aberrations in metabolism produce higher blood pressure is uncertain. Conversely, it is certain that a higher blood pressure commonly accompanies other metabolic risk factors (Shen et al., 2003). Blood pressure often is only moderately elevated in persons with the metabolic syndrome. Because blood pressure regulation is a complex process, it is not surprising that the metabolic connections between the various risk factors and blood pressure regulation are difficult to dissect. The frequency of association between blood pressure and those other risk factors justifies lumping them together as a multidimensional risk factor, however (Shen et al., 2003).

In the context of metabolic syndrome, it is necessary to return to the relationship between insulin resistance, blood pressure, and risk of CVD, specifically to emphasize that no more than 50% of patients with essential hypertension are insulin resistant but that this subset of patients is at greatest risk of CVD (Zavaroni et al., 1992). For example, patients with essential hypertension with electrocardiographic evidence of ischemic changes are somewhat glucose intolerant and hyperinsulinemic as compared with either a normotensive control group or patients with essential hypertension whose electrocardiograms are entirely normal (Jeppesen et al., 2000).

Changes in endothelial function those are likely to contribute further to increased risk of CVD also vary as a function of differences in insulin-mediated glucose disposal in patients with essential hypertension. For

example, the first step in the process of atherogenesis is the binding of mononuclear cells to the endothelium (Ross, 1986), and there is evidence of increased adherence of the cells isolated from patients with hypertension to the cultured endothelial cells (Chen et al., 1999). The relationship between insulin resistance and binding of isolated mononuclear cells to endothelium was similar in normotensive and hypertensive volunteers, because the more insulin resistant an individual, regardless of blood pressure status, the greater is the adherence of their isolated mononuclear cells to endothelium. The abnormality in the binding of isolated mononuclear cells to endothelium was seen only in the subset of patients with essential hypertension who were also insulin resistant.

Elevated plasma glucose

An increase in plasma glucose to above normal levels typically develops late in the course of the metabolic syndrome. Glucose elevation comes in several forms. The mildest form is called impaired glucose tolerance. This abnormality is detected by an oral glucose tolerance test, which is performed in persons with normal plasma glucose (<100mg/dl). Impaired glucose tolerance is defined as a plasma glucose level of 140 to 199 mg/dl two` hours after a 75-g oral glucose load. A second level of abnormality is impaired fasting glucose, which is defined as a fasting glucose of 100 to 125 mg/dl. It has been called pre-diabetes by the American Diabetes Association (Genuth et al., 2003). The third level is categorical hyperglycemia, which is designated as diabetes. Diagnosis of diabetes can be made with a fasting glucose level of 126 mg/dl or higher or a 2-hour glucose level of 200 mg/dl or higher (Genuth et al., 2003). Lesser increase in plasma glucose (impaired glucose tolerance/impaired fasting glucose) may not directly cause CVD, although they are associated with increased risk for CVD (Qiao et al., 2003). They also are strong risk factors for development of categorical diabetes (Unwin et al., 2002). All levels of increase-impaired glucose tolerance, impaired fasting

glucose, and diabetes-commonly are associated with other metabolic risk factors and must be considered as components of the metabolic syndrome (Alberti and Zimmet, 1998).

Although most insulin-resistant/hyperinsulinemic individuals do not become frankly hyperglycemic, they are at increased risk of developing type 2 diabetes. The role of insulin resistance as an important contributor to the development of human disease began with the evidence that resistance to insulin-mediated glucose disposal was a characteristic defect in patients with type 2 diabetes (Ginsberg et al., 1975). These initial observations have been confirmed on many occasions, and it has been shown that insulin resistance (or hyperinsulinemia as a surrogate measure of insulin resistance) is a powerful and independent predictor of the development of type 2 diabetes (Lillioja et al., 1993). Most insulin-resistant individuals maintain normal or near-normal glucose tolerance by secreting the large amounts of insulin needed to prevent the increase in plasma glucose and free fatty acid concentrations seen in patients with type 2 diabetes mellitus (Reaven, 1995). Type 2 diabetes only occurs when insulin-resistant individuals are no longer able to maintain the degree of compensatory hyperinsulinemia needed to maintain normal glucose homeostasis. Once hyperglycemia ensues, insulin-resistant individuals are at increased risk of developing the specific microangiopathic changes seen in patients with type 2 diabetes. Diabetic retinopathy, nephropathy, and neuropathy are the consequences of hyperglycemia, per se, not insulin resistance.

Prothrombic state

Several defects in the coagulation and fibrinolytic systems commonly are associated with other metabolic risk factors (Miller, 1994). These defects in aggregate can be called a prothrombic state. Examples of prothrombic defects include plasminogen activator inhibitor-1 (PAI-1), fibrinogen, and factor VII and platelet abnormalities. Theoretically, a prothrombic state could be

associated with CVD in several ways. Some of the prothrombic factors may be involved in the atherogenic process itself (Selwyn, 2003); these and others are likely to enhance the thrombotic response to acute plaque ruptures or plaque erosions (Libby, 2002). The pathogenesis of the prothrombic state varies, but because several aberrations in coagulation and fibrinolysis are common in persons who have the other metabolic risk factors, there is a growing view that these aberrations should be added to the list of metabolic risk factors.

Elevated PAI-1 levels, the principal inhibitor of fibrinolysis, are reported in many clinical and population studies of obese subjects, and they correlate with an abnormal pattern of obesity in men and women (Sakkinen et al., 2000) and other components of the metabolic syndrome inclusive of hyperinsulinemia, hypertension, high triglycerides, low HDL, and small LDL particles (Festa et al., 1999). In patients with type 2 diabetes, PAI-1 antigen localizes in endothelial and smooth muscle cells of the intima and medial layers of the arterial wall (Pandolfi et al., 2001). The accumulation of PAI-1 in arterial segments is accompanied by reduced plasma fibrinolysis. These findings provide insights into the higher clinical occurrence of thrombosis on ruptured plaques of patients with type 2 diabetes (Silva et al., 1995).

High levels of factor VII, a key component of the extrinsic coagulation cascade, may also contribute to a pro-thrombotic state, providing a potential mechanism for increased cardiovascular risk. This hypothesis was supported to some extent by the results of the Northwick Park Heart Study (NPHS; Meade et al., 1986). Similarly, the elevated levels of fibrinogen have been shown to be a strong and independent cardiovascular risk factor in prospective epidemiological studies (Ernst and Resch, 1993; Koenig, 2003). In addition, several *in vivo* studies have provided evidence that the loss of insulin's regulating action over platelet aggregation and activation in insulin resistance could contribute to the enhanced atherothrombotic risk associated with the metabolic syndrome (Lowe et al., 1980; Zahavi et al., 1981). Thus, it may be postulated that the metabolic syndrome has a permissive role on

thrombus formation, propagation and clot stability, and thereby increases the severity of the resultant ischemic event following plaque disruption and erosion.

Proinflammatory state

A final component of the metabolic syndrome is a proinflammatory state. In reference to CVD risk, this term is commonly used to indicate that atherogenesis is an inflammatory process. All of the steps in the development of atherosclerosis in one way or another are inflammatory. In classic terms, an inflammatory process has two major components: tissue injury and response to injury. Most of the metabolic risk factors such as lipid abnormalities, hypertension, hyperglycemia, and thrombotic factors potentially inflict direct injury on the arterial wall. Responses to arterial injury include infiltration of phagocytes and uptake of lipids, release of bioactive molecules by macrophages, and proliferation and collagen deposition by smooth muscle cells (Verma et al., 2003). These responses apparently elicit secondary inflammatory responses that include increased synthesis of acute phase reactants by the liver. One of these secondary products, C-reactive protein (CRP), provides a marker for the activity of the inflammatory process. Evidence is growing that persons with metabolic syndrome have high levels of CRP (Ridker et al., 2003).

The pro-inflammatory response includes an increased secretion of Interleukin-1β (IL-1β) and Tumor-necrosis factor-α (TNF-α), which then result in the release of the messenger cytokine, IL-6, especially from macrophages. IL-6, after engagement of its receptor on the liver, helps in the secretion and release of CRP and serum amyloid A (SAA). Recent evidence points to the role of vascular cells, such as smooth muscle cells, in the production of CRP. CRP mRNA and protein have been shown to be expressed in the cells of the lesion of magnitude more than that observed in plasma (Calabro et al., 2003; Kobayashi et al., 2003).

Cytokines, particularly IL-1, TNF-α, and IL-6, are the main inducers of the acute phase response (Baumann and Goldie, 1994). TNF-α is pro-inflammatory cytokine secreted by monocytes-macrophages, endothelial cells, and, to a large extent, by adipocytes. Several studies have shown that levels of TNF-α are important regulator of insulin sensitivity (Hotamisligil, 1993) and that neutralization of TNF-α improves insulin sensitivity in fa/fa rats but not in obese humans with diabetes (Arner, 2003). In human subjects, TNF-mRNA and protein correlate positively with body adiposity and decrease in obese subjects with weight loss. Insulin sensitivity was not studied, however (Dandona et al., 1998). Obesity is one of the major features of the metabolic syndrome. TNF-α levels also correlate strongly with BMI (Nieman, 1997). TNF-α is overexpressed in adipose and muscle tissue of obese individuals compared with tissues from lean individuals (Fernandez-Real and Ricart, 1999).

TNF-α and IL-6 decrease the activity of lipoprotein lipase in mice and cultured mouse adipocytes (Arner, 2003). TNF-α also promotes insulin resistance via decrease in insulin receptor tyrosine kinase activity, insulin receptor substrate-1 (IRS-1) phosphorylation, and GLUT4 synthesis/translocation (Hotamisligil et al., 1996). In contrast to IL-6, however, increased TNF-α secretion from adipose tissue has been shown only in rodents but not in humans.

HIGH-DENSITY LIPOPROTEIN IN METABOLIC SYNDROME

By definition, many subjects with the metabolic syndrome have low levels of HDL. Each of the features of the metabolic syndrome, including central adiposity, elevated plasma triglyceride, hypertension, and insulin resistance, is associated with a low concentration of HDL. Whether this reflects a causal relationship between these features and the concentration of HDL is not known. It is possible that a low level of HDL is just one of the several manifestations of same underlying condition, the metabolic basis of which

remains obscure. HDLs in subjects with the metabolic syndrome tend to be smaller and denser than normal (Chang et al., 1985). Whether this is a consequence of having the metabolic syndrome or a simple consequence of the low HDL level is not known. It is known, however, that the particle size of HDL correlates inversely with the concentration of plasma triglyceride. Subjects with plasma triglyceride concentrations more than 150 mg/dl tend to have predominantly smaller HDL (Yu and Mamo, 2000). When the triglyceride level falls below this value, there seems to be a quantum shift into a larger HDL size. Whether the elevated triglyceride in the metabolic syndrome accounts for the associated small size of the HDL particles is not known.

Several potentially pro-atherogenic forces operate in people with the metabolic syndrome. An increase in concentration of the remnants of triglyceride-rich lipoproteins has the capacity to deposit cholesterol in macrophages, converting them into foam cells (Goulinet and Chapman, 1997). There is also an increase in the concentration of small, dense LDLs, particles that are especially susceptible to oxidation (Lemieux et al., 2001) and subject to an enhanced uptake by macrophages. Subjects with the metabolic syndrome frequently manifest a pro-inflammatory state (Fielding and Fielding, 1995). Under normal conditions, pro-atherogenic forces such as these are opposed by HDLs. Not only do HDLs promote the efflux of cholesterol from cells, including macrophages (Mackness et al., 1993) but they also have antioxidant (Calabresi et al., 2002), anti-inflammatory (Rosenson and Lowe, 1998), and anti-thrombotic (Yuhanna et al., 2001) properties. They also stimulate endothelial nitric oxide production (Laws et al., 1997). In the metabolic syndrome, however, the HDL concentration is low and there is a reduced capacity to counter the cholesterol accumulation in macrophages and a decreased ability to prevent the oxidation of small dense LDL. This loss of the protection normally provided by HDL has the capacity

to amplify the already powerful pro-atherogenic forces that exist in the metabolic syndrome.

TRIGLYCERIDE AND LOW-DENSITY LIPOPROTEIN METABOLISM IN METABOLIC SYNDROME

Triglyceride-rich lipoprotein

Multiple factors contribute to altered metabolism of triglyceride and triglyceride-rich lipoproteins in the metabolic syndrome. Excess adiposity and insulin resistance foster increased hepatic production of VLDL and reduced intravascular catabolism and plasma clearance of VLDL and intestinally derived chylomicron particles. A major determinant of increased VLDL secretion in the metabolic syndrome is higher hepatic triglyceride content, derived in part from increased free fatty acid delivery from adipose tissue (Hotamisligil et al., 1995) and return of triglyceride-rich lipoprotein remnants to the liver. Changes in adipose tissue cytokines, including higher TNF-α (Yamauchi et al., 2001) and reduced adiponectin (Fisher and Ginsberg, 2002), also may increase hepatic VLDL production and impair peripheral clearance. Finally, insulin resistance and compensatory hyperinsulinemia can promote directly the increased hepatic secretion of VLDL particles (Ginsberg, 2002).

Intravascular catabolism and plasma clearance of triglyceride-rich lipoprotein involve complex interactions among lipases, apolipoproteins, lipid transfer proteins, and receptors (Krauss and Burke, 1982). Insulin resistance results in reduced activity of endothelial-bound lipoprotein lipase (LPL), which contributes to impaired triglyceride hydrolysis and uptake of chylomicron and VLDL lipids by muscle and adipose tissue. Insulin-resistant states also result in increased levels of apo-CIII, an inhibitor of LPL, and impaired apo-E-mediated receptor uptake of triglyceride-rich lipoproteins and their lipolytic remnants. Delayed postprandial clearance of diet-derived lipids in individuals with the metabolic syndrome can result from reduced intravascular catabolism of chylomicrons and competition with VLDL for

LPL activity. The net effect of changes in triglyceride-rich lipoprotein metabolism in the metabolic syndrome is an increase in plasma transport and prolonged plasma residence of these lipoproteins and their potentially atherogenic catabolic products. This in turn leads to increased levels of intermediate-density lipoproteins (IDL), the immediate metabolic precursors of LDL.

Low-density lipoprotein

At least seven subspecies of LDL can be distinguished on the basis of size and density. The subspecies, in turn, have been grouped into four subclasses that range from the largest, most buoyant (LDL-I) to the smallest and densest (LDL-IV) (Alaupovic, 2003). The differing subspecies of LDL have been shown to vary in lipid and carbohydrate composition and in conformation of apoB (Berneis and Krauss, 2002).

Multiple factors contribute to LDL heterogeneity, including differences in properties of their VLDL and IDL precursors and differences in intravascular transformation and catabolism. Evidence to date suggests that larger, more buoyant species arise from LPL-mediated lipolysis of smaller, relatively cholesterol-rich and triglyceride poor VLDL and IDL and by direct hepatic secretion (Karpe et al., 1993). In contrast, smaller, denser LDL species can derive from VLDL of progressively increasing size and triglyceride content by a process that involves LPL and hepatic lipase (HL) activities (Deckelbaum et al., 1984). Small, dense LDL also can arise by HL-mediated lipolysis of larger LDL and IDL after triglyceride enrichment of these particles through the action of cholesteryl ester transfer protein (Sakai et al., 1991). Cholesteryl ester transfer protein is not necessary for the production of small, dense LDL, however, as demonstrated by the presence of small triglyceride-enriched LDL particles in patients with genetic deficiency of cholesteryl ester transfer protein (Ehnholm et al., 1984). ApoE is also

believed to play a role in the conversion of VLDL to its smaller metabolic products, and loss of the protein is characteristic of most of the particles.

Several studies have shown a significant univariate relationship of reduced LDL peak particle size with increased risk of CVD. In most of these studies, however, the strength of this relationship was reduced substantially after adjustment for other risk factors [triglyceride (Stampfer et al., 1996) and total/HDL-C (Gardner et al., 1996)]. Evidence supports the selective benefit of lowering small, dense LDL particle concentrations on the risk of CVD (Miller et al., 1996). *In vitro* studies have suggested that small, dense LDL particles confer increased atherosclerotic risk through various mechanisms, including reduced LDL receptor affinity (Campos et al., 1996) and slower plasma clearance, increased transport into the sub endothelial space (Bjornheden et al., 1996), increased binding to heparan sulfate proteoglycans in the arterial wall, and increased susceptibility to oxidation (Chait et al., 1993).

HYPERTENSION AND METABOLIC SYNDROME

Within the metabolic syndrome cluster, there are several mechanisms through which one abnormality could favor the development of another. High blood pressure may modify peripheral tissue (skeletal muscle and fat) perfusion, either by microvascular rarefaction or through more functional subtle disturbances, thereby limiting vascular-to-tissue hormone and substrate exchange. Although attractive and supported by some experimental (Clark et al., 1995) and clinical (Baron et al., 1994) evidence, the vascular hypothesis for the development of insulin resistance has been challenged seriously by studies showing that in humans, physiologic hyperinsulinemia affects tissue perfusion only modestly (Utriainen et al., 1995) and that experimental improvement of tissue perfusion does not result in attenuation of skeletal muscle insulin resistance in patients with essential hypertension (Natali et al., 2000).

Alternatively, insulin resistance could raise blood pressure either by preventing the vasodilatory effects of the hormone or, via the attendant hyperinsulinemia, by upregulating the sympathetic and the antinatriuretic tone. The vascular effects of insulin are complex because they involve at least three mechanisms: hyperpolarization, nitric oxide-cyclic GMP, and ß-adrenergic stimulation-cyclic AMP. This pleiomorphic effect of insulin has been confirmed *in vitro*: in smooth muscle cells, insulin induces an increase in cAMP and cGMP that is receptor mediated and, for cGMP only, partly nitric oxide dependent (Trovati et al., 1995). At pharmacologic concentrations, insulin stimulates nitric oxide synthesis in human endothelial cells; this effect depends on the number of insulin receptors, their tyrosine kinase activity, and, downstream to the receptor, P13-kinase and Akt signaling (Zeng et al., 2000). Insulin's effect on the endothelium is not limited to the stimulation of nitric oxide synthesis. In rat mesenteric arteries, insulin vasodilatation is a transient phenomenon caused by a parallel slow-onset stimulation of endothelin synthesis (Misurski et al., 2001). The vascular net effect of insulin results from the combination of the two opposite vasoactive stimuli. Elegant experiments *in vitro* and in humans have shown recently that concurrent endothelin production masks the vasodilatory effect of low physiologic hyperinsulinemia (Verma et al., 2001). This could be the mechanism underlying the reduced vascular effect of insulin in insulin-resistant rats (Miller et al., 2002), which has an increased responsiveness to endothelin (Juul et al., 1996). The two effects have not only different time course but also different dose-response characteristics and may be differentially active depending on the associated conditions.

At physiologic concentrations, insulin enhances peripheral sympathetic outflow and directly desensitizes the sino-atrial node to the baroreflex control of heart rate (Muscelli et al., 1998). Mounting evidence also indicates that insulin, by trespassing (by transcytosis) the blood-brain barrier in the periventricular area, binds to neurons in the arcuate and paraventricular

nuclei, which then send inhibitory impulses to the vagus and excitatory impulses to the sympathetic nuclei (Davis et al., 1995). Overall, even in the absence of hypoglycemia, the cardiovascular system responds to acute insulin administration with a moderate, specific stress reaction. Of note is that the pattern of hemodynamic responses to euglycaemic hyperinsulinemia is maintained in obesity, an insulin-resistant state with a high-output, low-resistance hemodynamic pattern (Ferrannini, 1992). Similarly, physiologic hyperinsulinemia directly restrains renal sodium excretion by acting on the distal portions of the nephron (deFronzo et al., 1975). This action is preserved in individuals with insulin resistance of glucose metabolism (Muscelli et al., 1996) in whom chronic hyperinsulinemia might cause a rightward shift of the pressure-natriuresis curve (as documented in experimental animals (Fujiwara et al., 1999) and obese subjects (Rocchini et al., 1989).

MEDICAL TREATMENTS IN METABOLIC SYNDROME

There remains a need for medical intervention because about 50 percent of people with the metabolic syndrome do not reach targets without drugs. Furthermore, many people do not want to change their lifestyle or are unable to exercise. Therefore, drugs should be analyzed for their safety as well as effectiveness for prevention of the metabolic syndrome and associated cardiovascular diseases.

Medical treatment of diabetes in patients with the metabolic syndrome

So far drug intervention studies in pre-diabetes have been performed only in people with impaired glucose tolerance (IGT). No data from controlled prospective studies are available for subjects with impaired fasting plasma glucose (IFG). In subjects with IGT about one-third are suffering from the metabolic syndrome. In the US Diabetes Prevention Program (DPP) metformin (1-(diaminomethylidene)-3, 3-dimethyl-guanidine) was compared

with placebo and lifestyle modification. It is remarkable that metformin also reduced body weight by ~1Kg and had a beneficial effect on blood lipids but not on the blood pressure (deFronzo and Goodman, 1995).

Acarbose, an inhibitor of α-glucosidases of small intestine, delays the release of glucose from complex carbohydrates and thus reduces postprandial glucose excursion. The efficacy of treatment of postprandial hyperglycemia with acarbose in subjects with IGT was tested in a multi-national study. Such a treatment not only reduced conversion to diabetes by 36 percent but also reduced the incidence of newly diagnosed hypertension by 34 percent (Chiasson et al., 2003). Furthermore, a significant reduction in the triglycerides and excess weight was also observed. Interestingly, the therapeutic effect of acarbose on IGT and traits of the metabolic syndrome were associated with a significantly lower incidence of major cardiovascular events. Thus, both hypoglycemic drugs (acarbose and metformin) had beneficial effects in people with IGT and the metabolic syndrome.

The beneficial effects of metformin on parameters of the metabolic syndrome in clinical diabetes were confirmed by the UK Prospective Study Group (1998a). Metformin was the only drug in this mega-trial that significantly reduced cardiovascular events. It is still an open question why metformin, despite the fact that it achieved no stronger reduction in Hba1c than glibenclamide and insulin in the other arms of the group, was superior with respect to CVD. One explanation could be that it had therapeutic effects on overweight whereas the patients in the sulphonylurea and insulin groups gained weight.

Analysis of studies in type 2 diabetes with at-least one-year duration revealed, that acarbose improved several components of the metabolic syndrome: overweight, hypertension and hypertriglyceridemia. This was associated with a reduction of the incidence of myocardial infarction by 65 percent (Hanefeld et al., 2004).

The glitazones (pioglitazone, rosiglitazone) are insulin sensitizers that have additional beneficial effects on dyslipidemia and hypertension (Campbell, 2000; Raji et al., 2003). This is mainly due to a reduction of free fatty acids derived from intra-abdominal adipose tissue (Carey et al., 2002). Via activation of the nuclear receptor family PPARγ (peroxisome proliferator-activated receptors-γ), they are involved in glucose and lipid metabolism. Thus, they act on two major pathogenetic factors of the metabolic syndrome: insulin resistance and intra-abdominal obesity. Pioglitazone also activates PPARα. Their strong effect on insulin resistance has stirred great interest in their potential benefit in prevention of the metabolic syndrome and CVD. For pioglitazone, and less pronounced for rosiglitazone, a decrease in triglycerides, an increase in HDL-cholesterol and a reduction in the small dense LDL subfraction have been described. However, a minor increase in LDL-cholesterol occurs in patients with type 2 diabetes if glitazones treatment is introduced (Campbell, 2000). This is due to the increase in large buoyant LDL particles, which are considered to be less atherogenic (Freed et al., 2002). Furthermore, a reduction of blood pressure in patients with type 2 diabetes and hypertension has been described (Grossman, 2003).

A major effect of glitazones is on the free fatty acid (FFA) release from intra-abdominal fat. The FFA flux from intra-abdominal fat depots is an important determinant of hepatic insulin resistance and contributes to the development of non-alcoholic fatty liver disease (NFLD). Earlier clinical trials have shown that glitazones reduce steatosis hepatis (Promrat et al., 2004).

A reduction of the newly diagnosed diabetes was observed in some studies with ACE inhibitors and angiotensin-II-receptor antagonists (ARA) (Yusuf et al., 2001; Lindholm et al., 2002) and in a study with the statin pravastatin (Freeman et al., 2001). New studies are under way to test the potentials of combination therapy (rosiglitazone/ramipril and nateglinide/ valsartan) in subjects with IGT and IFG in the prevention of diabetes and cardiovascular complications. The surrogate markers so far available suggest

that glitazones should be effective in preventing CVD in clinical diabetes (Haffner et al., 2002).

In conclusion, oral hypoglycemics such as metformin, acarbose and insulin sensitizers are the drugs of first choice in subjects with pre-diabetes and type 2 diabetes that suffer from the metabolic syndrome. Furthermore, compounds used to treat conventional cardiovascular risk factors that improve insulin sensitivity and/or inhibit low-grade inflammation may be effective treatments for the metabolic syndrome.

Medical treatment of hypertension in patients with the metabolic syndrome

Recently four classes of antihypertensive drugs are commonly used as monotherapy or – in the majority of cases – in combination: angiotensin converting enzyme (ACE) inhibitors/angiotensin-II-receptor antagonists (ARA), beta-blockers, calcium channel blockers and diuretics. Besides the lowering of blood pressure, certain antihypertensives may have different pleiotropic effects on the pathophysiology of the metabolic syndrome:

ACE inhibitors such as Ramipril and ARAs like Losartan potassium have been shown to improve insulin resistance in many but not all studies. Accordingly, a reduction of newly diagnosed diabetes was reported in large prospective trials, with prevention of coronary heart disease as a primary outcome (Lindholm et al., 2002; Mann et al., 2003). Furthermore, ACE inhibitors/ARA reduces albumin excretion in diabetic patients with microalbumineria (Sica and Bakris, 2002; Mann et al., 2003). They have only marginal effects on lipids.

Beta-blockers, even the β_1-selective drugs, increase triglycerides, lower HDL-cholesterol and worsen insulin sensitivity. There are consistent data from prospective studies showing that they precipitate the onset of diabetes (Jacob et al., 1998). The use of atenolol, a β_1-selective beta-blocker, by the UK Prospective Diabetes Group (1998b) was associated with significantly higher

levels of Hba1c and weight gain, respectively, compared with the ACE inhibitor captopril.

Calcium channel blockers obviously have no significant effect on the diseases and pathophysiology of the metabolic syndrome. Diuretics such as hydrochlorothiazide and torasemide increase triglycerides and decrease HDL-cholesterol (Ferrari et al., 1991).

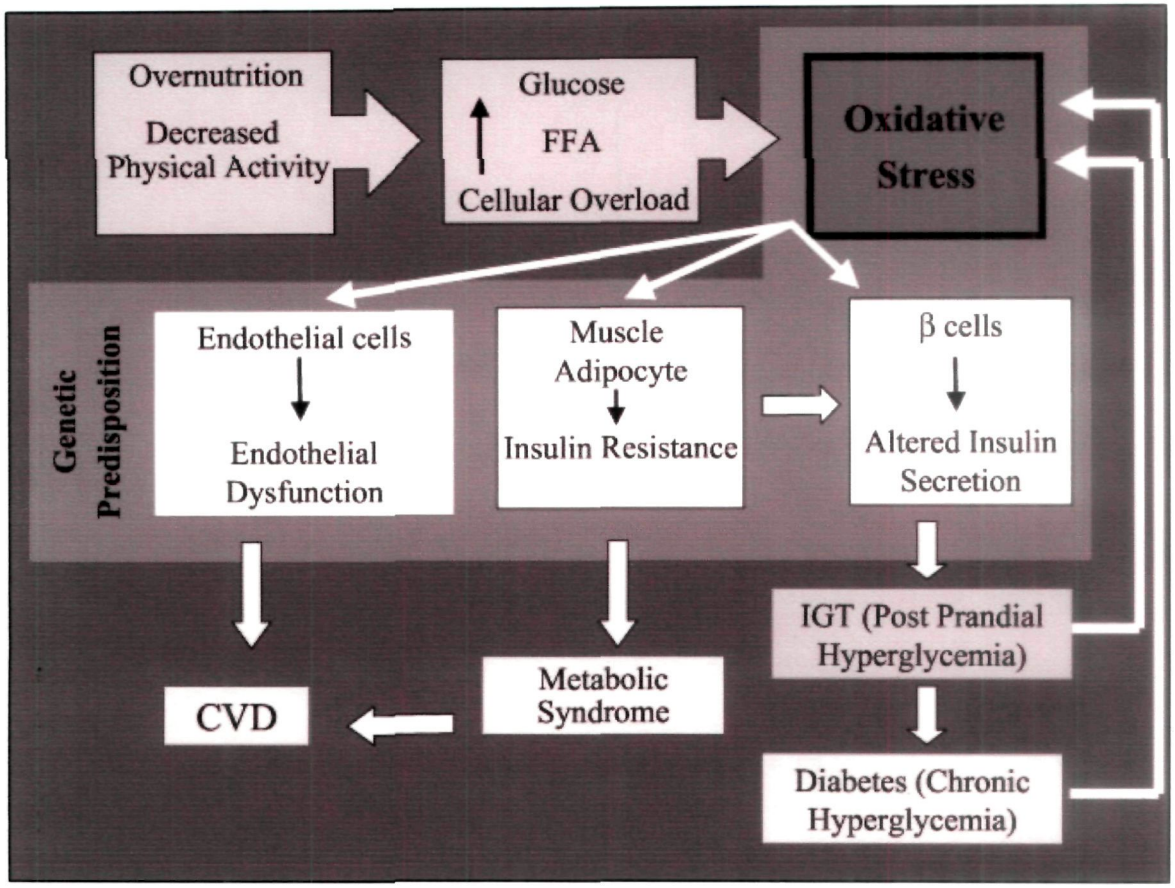
In patients with the metabolic syndrome without CVD, ACE inhibitors/ARA should therefore be the drugs of first choice, followed by calcium channel blockers. This is particularly relevant for young obese people with dyslipidaemia and/or pre-diabetes. In the majority of cases treated with ACE inhibitors/ARA, low doses of diuretics will be needed in the long term to achieve near-normal blood pressure levels. However, for patients with CVD and the metabolic syndrome the introduction of selective beta-blockers is of benefit, as shown by evidence-based prospective studies.

OXIDATIVE STRESS

Oxidative stress is the term used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable (Sies, 1991). Free radicals are reactive chemical species that contain one or more unpaired electrons. *In vivo* examples are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), superoxide radical ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), nitric oxide ($^{\cdot}NO$) and nitrogen dioxide ($^{\cdot}NO_2^-$); collectively described as reactive oxygen species (ROS). If not quenched by an antioxidant, these compounds will react with the nearest fat, protein, carbohydrate, RNA, or DNA molecule, altering its structure and function. Such interactions may also generate additional free radical centers, especially in polyunsaturated fatty acids. The nuclear DNA in every human cell receives an estimated 10,000 oxidative "hits" per day (Ames et al., 1993), indicating that cells are under constant bombardment from ROS. The antioxidant defense system includes enzymes such as

superoxide dismutase, glutathione peroxidase, and catalase; iron- and copper-binding extracellular proteins (such as albumin, transferrin, lactoferrin, hepatoglobin, and ceruloplasmin); antioxidants, such as vitamin C, vitamin E, quinones, glutathione, uric acid, bilirubin, and the carotenoids (Krinsky, 1992); and endogenous and exogenous polyphenolic compounds such as the flavonoids and lignans contained in fruits, vegetables, and legumes (Ratty and Das, 1988). Short term oxidative stress may occur in tissues injured by trauma, infection, heat radiation, hyperoxia, toxins, and excessive exercise (Halliwell et al., 1992). Alternatively, long term oxidative stress has been linked to CVD because oxidized low-density lipoproteins (LDLs) appear to be a prerequisite for foam cell formation and atherogenesis (Abby et al., 1993).

Of the five criteria of metabolic syndrome defined in NCEP/ATP III (Alexander et al., 2003), four (and notably hypertriglyceridemia, hypertension, hyperglycemia, and abdominal obesity) are independently characterized by elevated systemic oxidative stress (Bae et al., 2001; Redon et al., 2003; Oberley, 1988; Keaney et al., 2003). Furthermore, hypertriglyceridemia, hypertension, and obesity are each associated with increased production of superoxide anion via the nicotinamide adenosine diphosphate oxidase pathway (Hiramatsu and Arimori, 1988; Zalba et al., 2001; Brasier et al., 2002). In addition, hyperglycemia leads to increased formation of oxygen free radicals as a consequence of protein glycation and glucose autoxidation (Mezzetti et al., 2000). Initially, insulin resistance is compensated by hyperinsulinemia, through which a normal glucose tolerance is preserved.



FI: Oxidative Stress: Mediator of Metabolic Syndrome.

Deterioration to impaired glucose tolerance occur when insulin resistance increases further and/or the compensatory insulin secretory response decreases. An increase in insulin, free fatty acid (FFA) and/or glucose levels can raise ROS production and oxidative stress, as well as activate stress-sensitive pathways (Evans et al., 2003). This, in turn, can worsen both insulin action and secretion, thereby accelerating the progression to overt type 2 diabetes. Impaired glucose tolerance, i.e. postprandial hyperglycemia with fasting glucose in the normal range, is a risk factor for increased cardiovascular mortality (Ceriello, 2003a) and many studies show that postprandial hyperglycemia is associated with oxidative stress generation (Ceriello, 2003a). Repeated exposure to hyperglycemia and increased levels of FFA can lead to β-cell dysfunction that may become irreversible over time (Poitout and Robertson, 2002). In its initial stages, this damage is characterized by reversible defective insulin gene expression (Bruce et al., 2003). Glucose and lipid toxicity induce the gradual, time-dependent establishment of irreversible damage to cellular components of insulin production and therefore to insulin content and secretion (Prato, 2003). Oxidative stress is convincingly the mediator of such damage (Evans et al., 2003) as represented in Fig I (FI).

ANTIOXIDANTS AND METABOLIC SYNDROME

An antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate (such as proteins, lipids, carbohydrates and nucleic acids), significantly delays or prevents oxidation of that substrate" (Halliwell, 1996). The definition proposed by the Panel on Dietary Antioxidants and Related Compounds of the Food and Nutrition Board is that "a dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans" (Food and Nutrition Board, 1989).

When he proposed syndrome X, Reaven (Reaven, 1988) placed insulin resistance squarely at the heart of the syndrome, and considerable evidence exists to suggest that oxidative stress can contribute to insulin resistance (Evans et al., 2005). Thus, it may be instructive to examine some of the evidences about the possible benefits of antioxidants on insulin sensitivity. In a study of 1665 adults in the United States, serum concentrations of insulin were inversely associated with alpha-carotene, beta-carotene, lycopene, beta-cryptoxanthin, and lutein/zeaxanthin (Ford et al., 1999). In a study of 36 participants, those who were insulin resistant, determined with an insulin suppression test, had lower concentrations of alphacarotene, beta-carotene, lutein, alpha-tocopherol, and delta-tocopherol, but not beta-cryptoxanthin, zeaxanthin, lycopene, and gamma tocopherol, than patients without insulin resistance (Facchini et al., 2000). Among 182 participants of the Botnia study (Ylonen et al., 2003), plasma concentrations of beta-carotene were inversely associated with insulin resistance in men.

Unfortunately, little is known about how intakes of specific antioxidants may differ between people with and without metabolic syndrome. Among 8808 participants of the Third National Health and Nutrition Examination Survey (1988–1994) (Ford et al., 2003) in the United States, the use of vitamin or mineral supplements was not significantly different between participants with or without metabolic syndrome. Compared with participants who did not have metabolic syndrome, the intake of vitamin A was significantly lower among participants with the metabolic syndrome whereas the intake of vitamin C and E and carotenes was similar. In addition, participants with metabolic syndrome consumed fruits and vegetables less frequently than participants without this syndrome.

Several small studies suggested that vitamin E supplementation affects insulin action but not secretion (Paolisso et al., 1993; Paolisso et al., 1994). In 11 patients with type 2 diabetes mellitus, the use of 600 mg/day of

vitamin E for 3 months resulted in a reduction in the number of insulin receptors (Skrha et al., 1999). In a study of 80 overweight participants, vitamin E supplementation (800 IU/day for 3 months followed by 1200 IU/day for 3 months) improved insulin sensitivity during the first 3 months (Manning et al., 2004). Finally, in a randomized clinical trial, insulin sensitivity among 28 offspring of people with type 2 diabetes mellitus did not improve from using 800 IU/day of vitamin E for 12 weeks (McSorley et al., 2005). The effect of vitamin C supplementation on insulin parameters has also been studied. In a clinical trial in which participants were given 2 gm/day of vitamin C, insulin concentrations were decreased at 0.5 per hour but elevated at 2 hours during an oral glucose tolerance test (Johnston and Yen, 1994). In another trial of 40 patients with type 2 diabetes mellitus, the use of 0.5 gm/day of vitamin C resulted in decreased concentrations of insulin (Paolisso et al., 1995). In a study of 109 Japanese patients with type 2 diabetes mellitus, vitamin C supplementation (800 mg/d for 4 weeks) did not improve insulin resistance (Chen et al., 2006).

A number of cross-sectional studies have examined the associations between dietary patterns and metabolic syndrome or its components. Particularly germane to this work are studies that have examined the associations between dietary patterns characterized by increased antioxidant intake such as in a Mediterranean-style diet. This diet, in addition to "regular physical activity," emphasizes "abundant plant foods, fresh fruit as the typical daily dessert, olive oil as the principal source of fat, dairy products (principally cheese and yoghurt), and fish and poultry consumed in low to moderate amounts, zero to four eggs consumed weekly, red meat consumed in low amounts, and wine consumed in low to moderate amounts". Total fat in this diet is 25% to 35% of calories, with saturated fat at 8% or less of calories (Willett et al., 1995). The diet is often cited as beneficial for being low in saturated fat and high in monounsaturated fat and dietary fiber.

In a cross-sectional study of 2282 Greek men and women, participants who adopted a Mediterranean-style diet were less likely to have the metabolic syndrome than participants who did not (Panagiotakos et al., 2004). A few studies have suggested that a Mediterranean-style diet might favorably influence outcomes or physiologic abnormalities. Among Greek patients with an acute coronary syndrome who had metabolic syndrome, the adoption of a Mediterranean-style diet was associated with a 23% reduction in coronary risk (Pitsavos et al., 2003). In a randomized clinical trial of 180 Italian patients with metabolic syndrome, those who received the intervention that included the adoption of a Mediterranean-style diet showed favorable improvements in endothelial dysfunction and inflammatory markers (Esposito et al., 2004).

Several studies have suggested that the prevalence of metabolic syndrome differs by level of alcohol intake. Beverages such as wine are known to contain various antioxidants such as flavonoids. The studies examining dietary patterns or alcohol consumption were unable or did not isolate any possible effect of specific antioxidant intake on the study outcomes. However, Very few data are available about the associations between physiologic measurements of antioxidants, such as circulating concentrations, and metabolic syndrome from either cross-sectional studies or prospective studies. In addition, no studies have reported on the prospective associations between physiologic measurements of antioxidants and complications attributable to metabolic syndrome. No randomized clinical trials have been conducted among people with metabolic syndrome to test the effects of antioxidant supplementation on oxidative stress, antioxidant status, or other health outcomes.

VITAMIN C AS AN ANTIOXIDANT

Vitamin C is an important water-soluble antioxidant in biological fluids (Frei et al., 1990). Vitamin C readily scavenges reactive oxygen and nitrogen

species, such as superoxide, and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid (Halliwell, 1996), thereby effectively protecting other substrates from oxidative damage. Although vitamin C also reacts rapidly with hydroxyl radicals, it is nevertheless unable to preferentially scavenge this radical over other substrates (Niki and Noguchi, 1997). The reason for this is that hydroxyl radicals are extremely reactive and will combine indiscriminately with any substrate in their immediate environment at a diffusion-limited rate.

Two major properties of vitamin C make it an ideal antioxidant. First is the low one-electron reduction potentials of both ascorbate (282 mV) and its one-electron oxidation product, the ascorbyl radical (2174 mV), which is derived from the ene-diol functional group in the molecule (Halliwell, 1996). These low reduction potentials enable ascorbate and the ascorbyl radical to react with and reduce basically all physiologically relevant radicals and oxidants. For this reason, vitamin C has been said to be "at the bottom of the pecking order" and "to act as the terminal water-soluble small molecule antioxidant" in biological systems (Buettner, 1993). The second major property that makes vitamin C such an effective antioxidant is the stability and low reactivity of the ascorbyl radical formed when ascorbate scavenges a reactive oxygen or nitrogen species (Equation1). The ascorbyl radical readily dismutates to form ascorbate and dehydroascorbic acid (Equation 1 and 2), or is reduced back to ascorbate by an NADH-dependent semidehydroascorbate reductase (Wells and Jung, 1997). The 2-electron oxidation product of ascorbate, dehydroascorbic acid, can itself be reduced back to ascorbate by the glutathione-dependent enzyme, glutathione dehydroascorbate oxidoreductase [glutathione dehydrogenase (ascorbate), or glutaredoxin], or the NADPH-dependent selenoenzyme thioredoxin reductase (Wells and Jung, 1997). Alternatively, dehydroascorbic acid is rapidly and irreversibly hydrolyzed to 2, 3- diketogulonic acid (DKG) (Equation 3) (Halliwell, 1996).

$$AH^- \leftrightarrow A^{\cdot-} \leftrightarrow A \qquad \text{(Eq. 1)}$$

$$A^{\cdot-} + A^{\cdot-} \rightarrow AH^- + A \qquad \text{(Eq. 2)}$$

$$A \rightarrow DKG \rightarrow \text{oxalate, threonate, etc} \qquad \text{(Eq. 3)}$$

Equation 1 shows the reversible 1- and 2-electron oxidation of ascorbate (AH^-) to the ascorbyl radical ($A^{\cdot-}$) and dehydroascorbic acid (A), whereas equation 2 shows the dismutation of the ascorbyl radical to form ascorbate and dehydroascorbic acid. Equation 3 represents the hydrolysis of dehydroascorbic acid to DKG, which then decomposes to oxalate, threonate, and many other products. Vitamin C has been recognized and accepted by the US Food and Drug Administration (FDA) as one of 4 dietary antioxidants, the other 3 being vitamin E, the vitamin A precursor b-carotene, and selenium, an essential component of the antioxidant enzymes glutathione peroxidase and thioredoxin reductase.

VITAMIN E AS AN ANTIOXIDANT

Vitamin E is the generic term used to describe a group of at least eight compounds that exhibit the biological activity of α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. α-Tocopherol is the most active form of vitamin E, according to results from early animal growth assays (Food and Nutrition Board, 1989). In terms of antioxidant activity, results from more recent *in vitro* comparisons indicate that α-tocopherol is superior to γ-tocopherol as it usually exhibits less than 30% of the antioxidant activity of the α-tocopherol (Sies et al., 1993).

In plasma, vitamin E circulates in association with lipoproteins, mostly found in the LDL fraction under steady state conditions (Traber et al., 1993). In fact, α-tocopherol is the predominant antioxidant found in association with LDL; it is reported to be present at a molar ratio 6:1 (α-tocopherol to LDL) in well-nourished persons (Esterbauer et al., 1991). Recent epidemiologic

studies indicate that supplemental vitamin E consumption is inversely associated with the development of coronary artery disease in both men (Rimm et al., 1993) and women (Stampfer et al., 1993). Since oxidized LDL is implicated in the development and progression of atherosclerosis (Witztum and Steinberg, 1991) and α-tocopherol is known to limit LDL oxidation (Dieber-Rotheneder et al., 1991), it is attractive to speculate that the beneficial effects of α-tocopherol on coronary artery disease result from antioxidant protection of LDL. However, the effects of α-tocopherol on animal models of atherosclerosis are inconsistent (Verlangieri and Bush, 1992) despite continued protection of LDL against oxidation *ex vivo* (Keaney et al., 1994). Clearly then, the beneficial effects of α-tocopherol are not explained completely by its antioxidant protection of the LDL particle alone. α-tocopherol is incorporated into vascular tissue (Keaney et al., 1993) and may have important physiologic effects that are not directly related to the protection of LDL against oxidation *in vivo*. For example, α-tocopherol has been shown to influence leukocyte adhesion to endothelial cells (Faruqi et al., 1994), monocytes transmigration (Navab et al., 1991), and oxidant-mediated cytotoxicity (Hennig et al., 1987). Moreover, α-tocopherol is known to inhibit protein kinase C in vascular smooth muscle cells (Boscoboinik et al., 1991) and protein kinase C activation has been implicated in vascular disease due to diabetes (Tesfamariam et al., 1991) as well as oxidized-LDL (Ohgushi et al., 1993). Thus, these alternative effects of α-tocopherol have the potential to influence processes that are known to impair endothelium-dependent arterial relaxation.

Diabetic subjects and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of the antioxidant defense system and promoting the generation of free radicals (Hong et al., 2004). Vitamin E has been shown to decrease lipid peroxidation, inhibit platelet adhesion, aggregation, and smooth muscle cell proliferation, to exert its anti-inflammatory effect on

monocytes and to improve endothelial function (Harris et al., 2002). Animal studies have shown that vitamin E protects development of cholesterol-induced atherosclerosis by inhibiting protein kinase C activity in smooth muscle cells *in vivo* (Kartal et al., 2003). However, in spite of these evidences, the effect of α-tocopherol on blood pressure is controversial (Newaz and Nawal, 1998). Manifestations of vitamin E deficiency are exacerbated by deficiency of selenium (a necessary cofactor for glutathione peroxidase). This may be prevented by feeding other antioxidants (Farrell and Roberts, 1994). In cells, most of the vitamin E is situated in the membranes, adjacent to unsaturated fatty acids that are vulnerable to free-radical attack. Vitamin E is a potent chain-breaking antioxidant, scavenging oxygen radicals and terminating free-radical chain reactions (Burton and Traber, 1990). A number of studies have also suggested its role in prevention of cardiovascular diseases, cancer and Parkinson's disease as well (Hodis et al., 1995; Knekt, 1993; Stampfer et al., 1993).

Materials & Methods

MATERIALS

Alloxan monohydrate	Titan Biotech., India
Apo-B estimation kit	Giesse Diagnostics snc., Italy
Bovine serum albumin	Sigma Chemical Co., USA
Calcium chloride	SRL, India
CRP estimation kit	Calbiotech Inc., USA
Cupric chloride	SRL, India
Dimethylsulphoxide	Sigma Chemical Co., USA
Ethidium bromide	Sigma Chemical Co., USA
Ethylenediaminetetraacetic acid	Qualigens Fine Chemicals, India
Folin's reagent	BDH, India
Glucose estimation kit	Ranbaxy Diagnostic Div., India
HDL estimation kit	Ranbaxy Diagnostic Div., India
Heparin sodium salt	SRL, India
Histopaque 1077	Sigma Chemical Co., USA
Hydrogen peroxide	SRL, India
Low melting point agarose	Sigma Chemical Co., USA
Magnesium chloride	Qualigens Fine Chemicals, India
Nitrogen gas cylinder	Sigma Chemical Co., USA
Phenylacetate	SRL, India
Phosphate buffer saline	SRL, India
Phosphotungstic acid	SRL, India
Pyrogallol	SRL, India
Sodium chloride	Qualigens Fine Chemicals, India
Sodium hydroxide	BDH, India
Sulfuric acid	BDH, India
Tetramethoxypropane	SRL, India
TGs estimation kit	Ranbaxy Diagnostic Div., India
Thiobarbituric acid	Sigma Chemical Co., USA
TNF-α estimation kit	Immunotech SAS, France
Total cholesterol estimation kit	Ranbaxy Diagnostic Div., India
Triton X-100	BDH, India
Vitamin C (Ascorbic acid)	Central Drug House, India
Vitamin E (α-tocopherol)	Titan Biotech., India

All other chemicals were commercial products of analytical grade.

SUBJECTS

CHAPTER 1:

A total of 120 subjects were recruited on a consecutive basis for a screening program to check the prevalence of metabolic syndrome at the J.N. Medical College, A.M.U. Aligarh. Men and women aged 30-75 yrs participated in this study. All the volunteers, unaware of their health status and not observing any medication schedule for either diabetes or hypertension, were randomly selected and divided into three categories: Control subjects (those having normal FBG and BP), diabetic subjects (those having FBG level ≥110 mg/dl with normal BP), and hypertensive subjects (those having BP ≥130/80 mmHg with normal FBG). Demographic information and clinical history was obtained after getting informal consent of all the subjects. A measuring tape was used to measure the waist circumference of all volunteers at the high point of iliac crest and at normal minimal respiration. Blood pressure determination was made using a periodically calibrated mercury sphygmomanometer and measurements were performed after a 10-min rest. Two measurements were taken: the interval between the first and second was at least 20 min. The value used was the arithmetic mean of both determinations. Fasting blood specimens were obtained from each subject to determine the levels of FBG and serum was isolated for determination of total cholesterol (TC), TGs, HDL, CRP, TNF-α, apo-B levels, and all other parameters of metabolic syndrome (discussed in 'methods').

CHAPTER 2(A):

This study was based on 128 subjects who came for their regular check-up at J.N. Medical College, A.M.U. Aligarh. Men and women aged 30-75 yrs participated in this study. All the subjects were divided into four categories: Control subjects (those having normal FBG), diabetic subjects (those having

FBG ≥110 mg/dl and observing no medication; newly detected diabetes, including both type-1 and type-2 diabetes), diabetic subjects receiving metformin monotherapy (500 mg, orally administered once daily), and diabetic subjects receiving insulin monotherapy (mixture of intermediate and short acting insulin, with a dosage of 10 I.U. injected sub-cutaneously, twice a day). The diabetic subjects receiving either of the two monotherapies, as already prescribed by their physicians were on the regular treatment for past 5 months and not receiving any other medication for hyperglycemia. Demographic information and clinical history was obtained after getting informal consent of all the subjects. A measuring tape was used to measure the waist circumference of all volunteers at the high point of iliac crest and at normal minimal respiration. Fasting blood specimens were obtained from each subject to determine the levels of FBG and serum was isolated for determination of TGs, HDL, CRP, TNF-α, apo-B levels and all other parameters of metabolic syndrome (discussed in 'methods').

CHAPTER 2(B):

This study was based on 99 subjects who came for their regular check-up at J.N. Medical College, A.M.U. Aligarh. Men and women aged 30-75 yrs participated in this study. All the subjects were divided into four categories: Control subjects (those having normal BP), hypertensive subjects (those having BP ≥130/80 mmHg and observing no medication; newly detected hypertension), hypertensive subjects receiving ramipril monotherapy (5 mg, orally administered once daily), and hypertensive subjects receiving losartan monotherapy (50 mg, orally administered once daily). The hypertensive subjects receiving either of the two monotherapies, as already prescribed by their physicians, were on the regular treatment for past 5 months and not receiving any other medication for hypertension. Demographic information and clinical history was obtained after getting informal consent of all the

subjects. A measuring tape was used to measure the waist circumference of all volunteers at the high point of iliac crest and at normal minimal respiration. Blood pressure determination was made using a periodically calibrated mercury sphygmomanometer and measurements were performed after a 10-min rest. Two measurements were taken: the interval between the first and second was at least 20 min. The value used was the arithmetic mean of both determinations. Fasting blood specimens were obtained and serum was isolated from each sample for determination of TGs, HDL, CRP, TNF-α, apo-B levels and all other parameters of metabolic syndrome (discussed in 'methods').

CHAPTER 3:

Male albino rabbits weighing between 1.0 and 1.5 kg were used and maintained on commercial diet and water *ad libitum*. They were acclimatized to the laboratory conditions (for 2 weeks) before carrying out any experimental work and kept under the controlled conditions of temperature (37±2°C), and 12 hrs light/dark cycle throughout the experiment. The Experimental Ethical Committee for Animal Research approved the protocols used in this study.

Induction of diabetes

Diabetes was induced by administering intravenous injection of 5% solution of alloxan monohydrate (80 mg/kg body weight), dissolved in cold normal saline, to overnight fasted rabbits through their marginal ear vein. Fasting blood glucose (FBG) was checked regularly up to the development of stable hyperglycemia after three weeks of alloxan administration. The diabetic state was confirmed when FBG concentration exceeded 200 mg/dl.

Animal treatment

A total of 12 rabbits were distributed into four experimental groups of three rabbits per group: non-diabetic (control, G1); diabetic (G2); diabetic supplemented with 150 mg/kg body weight of vitamin C (ascorbic acid), dissolved in water (G3) (Sokoloff et al., 1967) and diabetic supplemented with 1000 I.U./kg chow of vitamin E (α-tocopherol), dissolved in olive oil (G4) (Keaney et al., 1996), administered daily as an oral supplementation via gavage for a period of two weeks. The control group however, received olive oil only, so as to rule out the effects of olive oil alone. Thereafter, 5 ml fasting blood was collected from each animal by piercing the marginal ear vein to determine their FBG and serum was isolated from each sample for the determination of TGs, HDL, CRP, TNF-α, apo-B levels and all other parameters of metabolic syndrome (discussed in 'methods').

METHODS

Estimation of Total Cholesterol (TC)

Total cholesterol in the serum was determined by the cholesterol-esterase-oxidase-peroxidase method (Richmond, 1992). The assay was based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters in serum. Cholesteryl esters in the sample were first hydrolyzed by cholesterol esterase into cholesterol, which was then oxidized by cholesterol oxidase to yield hydrogen peroxide and the corresponding ketone product. Hydrogen peroxide was then detected by using ADHP (10-acetyl-3,7-dihydroxyphenoxazine), a highly sensitive and stable probe for hydrogen peroxide. In the presence of horseradish peroxidase, ADHP reacts with hydrogen peroxide with a 1:1 stoichiometry to produce highly fluorescent resorufin.

The assay procedure was followed as per the directives of Ranbaxy Diagnostic Div., India. Typically, 50 µl of cholesterol standards as well as serum samples were added first of all, in the wells of a microtiter plate. Thereafter 50 µl of the assay cocktail was added to each well. This assay cocktail was prepared immediately before the start of experiment and included 4.745 ml of assay buffer (0.1 M potassium phosphate pH 7.4, containing 50 mM sodium chloride and 5 mM cholic acid), 150 µl of cholesterol detector (ADPH), 50 µl of horseradish peroxidase, 50 µl of cholesterol oxidase, and 5 µl of cholesterol esterase. This was followed by a further incubation for 30 min at room temperature in dark and the fluorescence was recorded using excitation wavelengths of 565-580 nm and emission wavelengths of 585-595 nm.

Estimation of Triglycerides (TGs)

Triglycerides levels in the serum were estimated by modified enzymatic method (Trinder, 1969) using color reaction to produce a fast, linear, end point reaction (Fossati and Prencipe, 1982; McGowan et al., 1983). In this assay, triglycerides were first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was then phosphorylated by ATP with the help of glycerol kinase forming glycerol-1-phosphate. Glycerol-1-phosphate was then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyzes the coupling of hydrogen peroxide with 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that showed an absorbance maximum at 540 nm.

The assay procedure was performed as per the directives of Ranbaxy Diagnostic Div., India. To begin with, 10 µl each of triglyceride standards as well as serum samples were added to the wells of a microtiter plate. The reaction was initiated subsequently by the addition of 150 µl of diluted enzyme buffer, a solution of triglyceride enzyme mixture in 15 ml of 50 mM sodium phosphate buffer (pH 7.2). This was followed by incubation at room temperature for 15 min and the absorbance was read at 540 nm. The increase in absorbance at this wavelength was directly proportional to triglyceride concentration of the sample.

Estimation of High density lipoprotein (HDL)

HDL quantification is based on the polyethylene glycol (PEG-6000) precipitation method in which HDL concentrations are determined using cholesterol esterase/cholesterol dehydrogenase reagent (Viikari, 1976). In this reaction, NAD was reduced to NADH. The optical density of thus formed NADH at 340 nm was directly proportionate to the concentration of HDL in the sample.

The assay procedure was performed as per the directives of Ranbaxy Diagnostics Div., India. The serum samples were first mixed with precipitation reagent (20 µl each) and allowed to centrifuge at 9,500 rpm for 5 min. The supernatant thus obtained (HDL) was diluted with the assay buffer in a ratio of 1:4. Thereafter, 50 µl each of standards and HDL were pipetted into the wells of a microtiter plate. This was followed by addition of working reagent (60 µl) in each well to initiate the reaction. The working reagent was prepared by mixing 50 µl assay buffer, 18 µl NAD solution, and 1 µl enzyme mix, (as provided with the kit). The whole reaction set-up was then incubated for 30 min at room temperature and the absorbance was recorded at 340 nm.

Estimation of Low density lipoprotein (LDL)

LDL concentration was calculated using the Friedewald formula (Friedewald et al., 1972) as follows:

LDL = [Total cholesterol – {Triglycerides/5 + High density lipoprotein}]

The concentrations of previously estimated TC, TGs and HDL were used for this calculation.

Estimation of CRP

Estimation of CRP is based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA). Assay procedure was followed as per the directives of the kit provided by Calbiotech Inc., USA and the details are given below:

The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the CRP molecule. This mouse monoclonal anti-CRP antibody was used for solid phase immobilization (on the microtiter wells). A goat anti-CRP antibody was present in the antibody-enzyme (horseradish peroxidase) conjugate solution. The serum samples as well as calibrators (CRP standards), 25 µl each, were allowed to react simultaneously with the two antibodies (100 µl), resulting in the CRP

molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 15 min incubation at room temperature, the wells were washed with 300 µl of wash buffer, a solution of buffered saline in distilled water, to remove unbound-labeled antibodies. Thereafter, 100 µl of working substrate solution, containing tetramethylbenzidine and hydrogen peroxide was added and incubated for 15 min, resulting in the development of blue color. The reaction was stopped with the addition of Hydrochloric acid (50 µl), that changed the color to yellow. The concentration of CRP was directly proportional to the color intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm.

Estimation of TNF-α

Estimation of TNF-α is also based on the principle of ELISA. Assay procedure was followed as per the directives of the kit provided by Immunotech SAS, France and the details are given below:

At first, 100 µl of conjugate containing the first monoclonal antibody anti-TNF-α in presence of the second anti-TNF-α monoclonal antibody linked with alkaline phosphatase was added to the wells of a microtiter plate. Serum samples as well as the calibrators (TNF-α standards), 100 µl each, were then allowed to react simultaneously with the conjugate following incubation for 2 hours at room temperature. After incubation, the wells were washed carefully with the wash solution diluted in distilled water. The bound enzymatic activity was then detected by addition of 200 µl of substrate dissolved in diethanolamine-HCl buffer which was further incubated in dark for 45 min. The reaction was stopped with the addition of 50 µl stop solution containing sodium hydroxide. The color intensity was read at 405 nm, and was proportional to the TNF-α concentration in the sample or calibrator.

Estimation of apo-B

Turbidimetric test was used for the measurement of apo-B in serum. The assay procedure was followed as per the directives of the kit provided by Giesse Diagnostics snc., Italy and the details are given below:

A goat serum anti-apo-B antibody was first diluted in the Tris buffer in a ratio of 1:41 to make the working reagent. At the same time, serum samples as well as calibrators (apo-B standards) were also diluted with saline solution in a ratio of 1:15. Thereafter, I ml of working reagent and 100 µl of diluted serum sample or calibrator were pipetted into cuvette and incubated for 10 min at room temperature. The blanks devoid of samples were also worked out in the same manner and absorbance was read on a spectrophotometer with a 340 nm filter.

Determination of FBG

The most widely used method for the determination of blood glucose was proposed by Trinder in 1969. In this reaction, glucose oxidase reacts with glucose to form gluconic acid and hydrogen peroxide. The hydrogen peroxide is further splitted by peroxidase and reacts with 4-aminoantipyrine and phenol to produce a colored complex. The intensity of the coloration, read at 505 nm, is proportional to the concentration of glucose in the sample.

The assay procedure was followed as per the directives of Ranbaxy Diagnostic Div., India. Briefly, 10 µl of the fasting blood samples were mixed with 1 ml of working solution (containing 6.7 U/ml of glucose oxidase, 6.2 U/ml of horseradish peroxidase, 0.2 mM of 4-aminoantipyrine, 8 mM of phosphate buffer and 86 mM of phenol). The blank and standard solutions were also prepared simultaneously by adding 10 µl of distilled water and 10 µl of standard glucose (100 mg/dl) to the 1 ml of working solution. All the tubes were mixed well and incubated at room temperature for 30 min. Blank solution was used to set the spectrophotometer and absorbance was read at 505 nm.

Estimation of MDA

MDA in serum samples was determined fluorimetrically by thiobarbituric acid reaction (TBA). 0.2 ml of serum was taken in a stoppered centrifuge tube, made up to 1 ml with 0.9% sodium chloride solution, treated with 4 ml of N/12 sulfuric acid and shaken gently. 0.5 ml of 10% phosphotungstic acid was then added and mixed well followed by centrifugation at 3000 rpm for 10 min. The supernatant thus obtained, was discarded and the sediment was treated with 2 ml of N/12 sulfuric acid and 0.3 ml of 10% phosphotungstic acid followed by centrifugation again for 10 min. Hence obtained supernatant was further discarded and to the sediment, 4 ml of distilled water and 1 ml of TBA reagent were added. Thereafter, 4 ml of distilled water and 4 ml of 0.5 nmol/l of tetramethoxypropane were taken as blank and standards respectively and 1 ml of TBA reagent was added to both the tubes. All the tubes were then incubated at 95°C in a boiling water bath for 60 min. At the end of this, the tubes were removed, cooled and treated with 5 ml of n-butanol. Tubes were mixed vigorously and centrifuged at 3000 rpm for 15 min. The upper n-butanol layer was then taken for fluorimetric measurement at 553 nm emission and 515 nm excitation. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum (Kunio, 1984).

Lowry method for protein estimation

The Lowry method for determination of protein concentration is one of the most venerable and widely-used protein assays. It was first described by Lowry and coworkers in 1951. The method is based on both the biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^{+}, which reacts with the Folin's reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids.

Briefly, 0.5 ml serum was mixed with 5 ml of freshly prepared copper reagent. To this solution, 0.5 ml of Folin's reagent was added after 10 min. This resulted in the development of blue color, which is partly due to the tyrosine and tryptophan content of protein. The intensity of the color produced was directly proportional to the concentration of protein in the serum sample which was measured spectrophotometrically at 660 nm using BSA as a standard.

Assay of SOD activity

The assay method was based on monitoring the inhibition of auto-oxidation of pyrogallol by SOD (Marklund and Marklund, 1974). The assay medium in a final volume of 3 ml consisted of 50 µl sample (serum) and 2.85 ml of 0.05 M tris-succinate buffer, pH 8.2. After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. Blanks devoid of sample were worked up in the same manner and run simultaneously. One enzyme unit is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Enzyme activity was expressed as units per mg of protein.

Estimation of catalase

The assay principle is summarized in the reaction scheme below:

$$\underset{\text{Absorbance at 240 nm}}{2H_2O_2} \xrightarrow{\textit{catalase}} \underset{\text{No absorbance at 240 nm}}{2H_2O + O_2}$$

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H_2O_2) (Aebi, 1984). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml of 30 mM H_2O_2 and 50 µl ml sample. One enzyme unit is

defined as the amount of enzyme decomposing 1 μM H_2O_2 per min at 25°C. Enzyme activity was expressed as units per mg of protein/min.

Small dense LDL-C Assay

The details and validation of this experiment have been described elsewhere (Hirano et al., 2003). In brief, the precipitation reagent (0.1 ml) containing 150 U/ml of heparin sodium salt and 90 mM magnesium chloride was added to 0.1 ml of serum sample and the samples were incubated for 10 min at 37 °C after mixing. Each sample was placed in an ice-bath and allowed to stand for 15 min after which the precipitates were collected by centrifugation at 15000 rpm for 15 min at 4 °C. The precipitates were always packed tightly at the bottom of the tube and the supernatant was clear. This heparin-Mg^{2+} supernatant contain sdLDL cholesterol with no influence of other lipoproteins (Hirano et al., 2004). Nitrogen gas was bubbled through the supernatant collected in separate tubes, which were then closed tightly with parafilm and kept at 4 °C in dark to protect it from oxidative modification.

Oxidation of small dense LDL

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989) for LDL oxidation. 10 μl of supernatant containing sdLDL, collected above, oxygenated PBS and 32 μl of 1 mM cupric chloride was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored (Ashton et al., 2000) continuously at 37 °C on a UV spectrophotometer at 234 nm.

Assessment of oxidative damage (Lipid peroxidation)

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the

dark, and reading the absorbance at 365 nm. The concentration of lipid peroxides was calculated (el-Saadani et al., 1989) using a molar absorption coefficient of 2.46×10^4 $M^{-1}cm^{-1}$. In principle, this assay makes use of the oxidative capacity of lipid peroxides to convert iodide to iodine, which can be measured photometrically at 365 nm. Concentrations of lipid peroxides as low as 1 nmol/ml could be measured by this procedure.

Measurement of arylesterase activity of PON-1 using phenylacetate as a substrate

Initial rates of hydrolysis were determined spectrophotometrically at 270 nm in a power wave 200 microtiter scanning spectrophotometer. The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM calcium chloride dissolved in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum. The extinction coefficient at 270 nm for the reaction was 1307 mol/l/cm. One unit of arylesterase activity is equal to 1 µmol of phenylacetate hydrolyzed per min (de Geest et al., 2000).

DNA damage determination by alkaline comet assay

One ml of venous blood sample served for DNA damage assessment using alkaline comet assay. Lymphocyte isolation for the comet assay was performed using the Histopaque 1077. 1ml of heparinised blood was carefully layered over 1 ml histopaque and centrifuged for 35 min at 500 × *g* and 25°C. The interface band containing lymphocyte were washed with phosphate buffered saline (PBS) and then collected by 15 min centrifugation at 400 × *g*. The resulting pellets were resuspended in PBS to obtain approximately 20,000 cells in 10 µl.

Comet assay was performed according to Singh et al. (1988) with the slight modifications. Essentially, 100 µl of 0.5% normal melting agarose was pipetted onto frosted microscope slides and allowed to solidify under a coverslip, which was then carefully removed. Approximately, 20,000 cells

(10 µl) were suspended in 75 µl low melting agarose gel (LMPA); the cell suspension was rapidly pipetted onto the first agarose layer, and gently spread by placing a coverslip on top. This was allowed to solidify on an ice tray for 5 min. After removal of the cover slip, the slide was immersed in freshly prepared lysing solution (2.5 M sodium chloride, 100 mM EDTA, and 10 mM Tris, with 1% Triton X-100, and 10% DMSO) prepared just before use and incubated overnight at 4°C. The slides were removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank. The tank was filled with fresh, cold electrophoresis solution (1 mM EDTA and 300mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali-labile damage before electrophoresis.

Electrophoresis was conducted at 4 °C for 30 min using 25 V and a current of 300 mA. Following electrophoresis, the slides were washed thrice in Tris buffer (0.4 M, pH 7.5) to neutralize the excess alkali. Finally the slides were stained with 75 µl ethidium bromide (20 µg/ml) and covered with a coverslip. The slides were then placed in a humidified chamber to prevent drying of the gel and analyzed the same day. Slides were scored using an image analysis programme (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to an Olympus (C×41) fluorescent microscope and an integrated camera (equipped with a 540-560 nm excitation and 590 nm barrier filter). Comets were scored at 100x magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocyte DNA damage was tail length (migration of DNA from the nucleus, µmeters) and was automatically generated by Komet 5.5 image analysis programme.

Statistical analysis

Results were expressed as mean ± S.D. for all continuous variables. The qualitative data were expressed in percentages. Differences between the control, diabetic, hypertensive, and metformin/insulin/ramipril/losartan treated groups as well as antioxidant supplemented groups were assessed using ANOVA followed by student's t-test. Associations between DNA damage and parameters of metabolic syndrome along with the parameters of NCEP/ATP III were evaluated by Pearson's correlation test. All tests were two-tailed and p-value less than 0.05 were considered to be statistically significant. All the analysis was performed with sigma statistical software, Sigmastat 3.5 and Origin 6.1.

Results

Chapter 1

Prediction of Metabolic Syndrome in Diabetic and Hypertensive Subjects

The metabolic syndrome is a combination of several factors which may share a common aetiology and each of which is a risk factor contributing to CVD. In the past few years the concept of metabolic syndrome has been a permanent topic of debate, particularly concerning its pathogenesis and clinical usefulness, and today still no consensus has been reached (Grundy, 2006). This is partially reflected by the use of several definitions, based on different diagnostic criteria, among them the definition proposed by the WHO, NCEP/ATP III, and that of EGIR are all the rage (Alberti and Zimmet, 1998). The various definitions have been shown to identify different individuals hindering comparisons between different studies and different populations. The NCEP/ATP III definition has however been found to be the most straightforward to implement because the five criteria (abdominal adiposity, hypertension, hypertriglyceridemia, low HDL, and hyperglycemia) were clearly defined. Although not included in the diagnostic criteria, an increase in the secretion of apoB-containing lipoproteins and pro-inflammatory cytokines may also contribute to the increased incidence of diabetes and CVD (Eckel, 2007). It has also been reported previously that the metabolic syndrome is well characterized by the presence of smaller, denser lipoprotein particles that increase their susceptibility to oxidative modification and a diminished serum paraoxonase (PON-1) activity that is a major determinant of the antioxidant capacity of HDL. These may be contributory factors to the increased presence and severity of coronary diseases in such patients (Garin et al., 2005). In general, the greater the number of metabolic syndrome components, the greater is the risk for these outcomes.

The newly detected diabetic and hypertensive subjects were consequently chosen for this study, to identify their risk of developing metabolic syndrome by estimating the parameters proposed by NCEP/ATP III along with the other risk markers.

1.1: Anthropometric observations

As earlier reports have proposed the involvement of elevated blood glucose and increased blood pressure in metabolic syndrome (Grundy et al., 2004), both diabetic and hypertensive subjects were included in the present work. Table.1.1 represents the anthropometric characteristics of control (C), diabetic (D) and hypertensive subjects (HT). It clearly depicts the elevated levels of fasting blood glucose (FBG) and blood pressure (BP) along with the records of age, family history and waist circumference of all subjects.

Several reports have suggested that prevalence of metabolic syndrome is associated with age. In the third National Health and Nutrition Examination Survey (NHANES III) performed in the USA, the prevalence of metabolic syndrome increased from 6.7% among participants of 20-29 years of age to 43.5% for 60-69 year-olds and was 42.0% for participants of 70 years or older (Alexander et al., 2003). Similar pattern was observed in this study as well. Our observations (table.1.1) illustrate that most of the diabetic and hypertensive subjects were from the age group of 50-59 years.

The effect of generalized obesity is also extremely important so much so that, in populations where obesity is more common in women than in men, the prevalence of metabolic syndrome is likely to be higher in women than in men. This trend can be observed in Indian, Iranian and Turkish populations (Onat et al., 2002; Azizi et al., 2003; Gupta et al., 2003; Ramachandran et al., 2003; Ozsahin et al., 2004). However, the pattern observed in the present study was in-a-way opposite as it showed higher waist circumference of men as compared to women. Besides, table.1.1 also suggests that the waist circumference of both hypertensive and diabetic subjects is always greater than that of control subjects.

The available data describing the role of genetic factors in determining the prevalence of metabolic syndrome are limited and many findings have yet to be replicated in other populations (Corella and Ordovas, 2004). The data from this study suggests, subjects having a family history of

either diabetes or hypertension are at a higher risk of developing the same disease and hence metabolic syndrome (table.1.1).

1.2: Lipid profile

The dyslipidemia typically found in subjects with the metabolic syndrome includes an elevated concentration of plasma TGs, a fraction of LDL in which the particles are smaller and denser than normal, and a low concentration of HDL cholesterol (Alberti and Zimmet, 1998). Fig.1.2 depicts the concentration of total cholesterol in all the participants of this study. It perceives that the average cholesterol of diabetic subjects (D) was maximum followed by hypertensive (HT) and control subjects (C). This result was consistent to the previous reports that have suggested an alteration in cholesterol metabolism during insulin deficiency (Young et al., 1988). It is known that in type 2 diabetic subjects, cholesterol absorption efficiency is low (Simonen et al., 2002), whereas the synthesis of cholesterol is high (Abrams et al., 1982). This could be a primary factor in modifying cholesterol metabolism (Young et al., 1988).

The figure also demonstrates the concentration of TGs in all study groups. Mean TGs were highest in hypertensive subjects (HT), followed by diabetic subjects (D) and lowest in control subjects (C). A meta-analysis of 17 prospective, population-based studies found that an increase in plasma triglyceride of 1 mM, or 89 mg/dl, was associated with an increased risk of CVD (Hokanson and Austin, 1996). The Physician's Health Study found that the relative risk of myocardial infarction increased with increasing postprandial triglyceride concentrations (Stampfer et al., 1996). A study by Jeppesen et al. (1998) has suggested that high plasma triglycerides were associated with an increased risk of ischemic heart disease. Evidence also exists that a combined measurement of plasma triglyceride and HDL, provides a strong index of risk of CVD (Gaziano et al., 1997). Eventually, measurement of HDL was also performed in this study and is demonstrated

in the same figure. No significant variation in HDL levels was observed among the three categories. Moreover, the values observed for diabetic (D) and hypertensive subjects (HT) were almost always similar. In addition, the LDL levels among control, diabetic, and hypertensive subjects are also illustrated and it is deduced that the concentration of LDL was highest in hypertensive subjects (HT), followed by diabetic (D) and control subjects (C).

1.3: sdLDL oxidation

A lipoprotein profile characterized by the predominance of small, dense LDL particles is one of the key elements of atherogenic dyslipidemia in metabolic syndrome (Krauss and Siri, 2004). *In vitro* studies have suggested that sdLDL particles confer increased atherosclerotic risk due to their increased susceptibility to oxidation (Chait et al., 1993). It was discovered that a combination of heparin and magnesium precipitate a part of LDL which remained in the supernatant. This fraction was identified as sdLDL and found very similar to the one isolated by ultracentrifugation. The supernatant obtained thus, was used as a source of sdLDL in the present study. It was oxidized in-vitro using cupric chloride (details given in 'methods') and the kinetics was observed in terms of peak time. The results presented in fig.1.3 indicate the earliest peak of oxidation of sdLDL among hypertensive subjects (HT) followed by diabetic (D) and control subjects (C). Thus, suggesting hypertension as a major risk to atherogenesis and metabolic syndrome. This result was in agreement with the previous reports that have found an inverse relationship between the lag time of in vitro LDL oxidation and the severity and progression of coronary atherosclerosis (Regnstrom et al., 1992) suggesting the enhanced susceptibility to oxidation may underlie the excess vascular disease observed in patients with diabetes. This is also consistent with the studies showing that the mean lag time of sdLDL oxidation is lower in patients with diabetes than in controls (Dimitriadis et al., 1995; Yoshida et al., 1997).

Parameters	C (n=67)	D (n=37)	HT (n=16)
Males (n) Females (n)	39 28	28 9	9 7
FBG (mg/dl)	98 ± 1.3	172 ± 1.8	108 ± 2.1
BP (mm Hg) (Sys) (Dia)	 113 ± 1.5 65.3 ± 1.3	 115 ± 2.1 65.8 ± 2.0	 131 ± 2.2 79.8 ± 2.5
Age prevalence (Yrs)	30-39	50-59	50-59
WC (cm) Males Females	 86 ± 1.5 75 ± 1.6	 105 ± 1.6 95 ± 1.6	 104 ± 1.9 93 ± 2.4
Family history Positive Negative	 12% 88%	 40 % 60 %	 56 % 44 %

Table.1.1: Anthropometric observations in diabetic and hypertensive subjects.

The blood glucose level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 120 mg/dl. The blood pressure (BP) and waist circumference (WC) for each subject was measured by the standard techniques (as described in 'methods'). Results are expressed as mean ± S.D. or percentage, wherever applicable.

C Control group
D Diabetic group
HT Hypertensive group

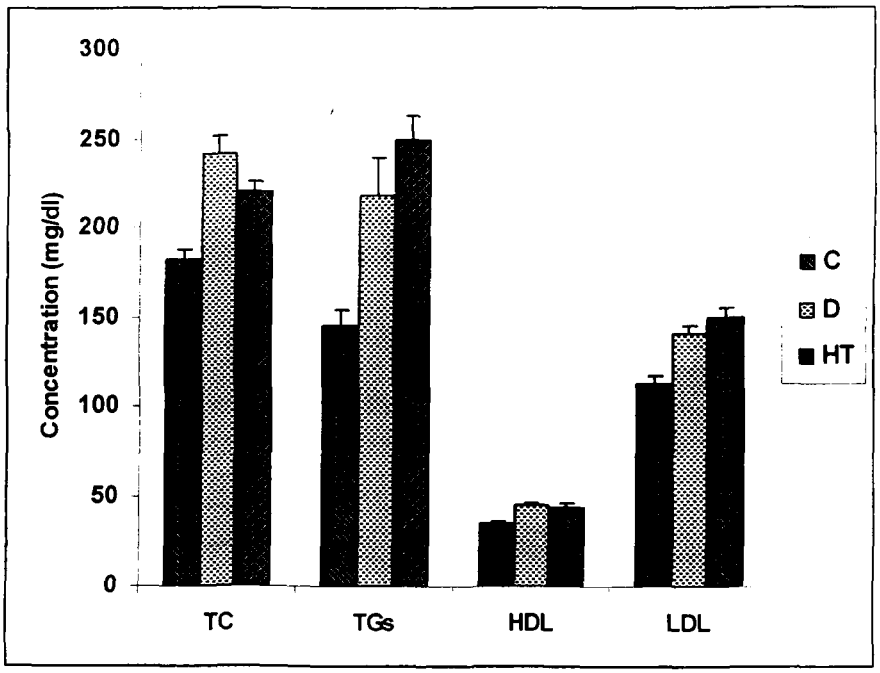


Fig.1.2: Lipid profile of diabetic and hypertensive subjects.

The concentration of serum TC, TGs, and HDL was measured by an enzymatic colorimetric test using commercial kits from Ranbaxy Diagnostic Div., India. LDL concentration was calculated using the Friedewald formula. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

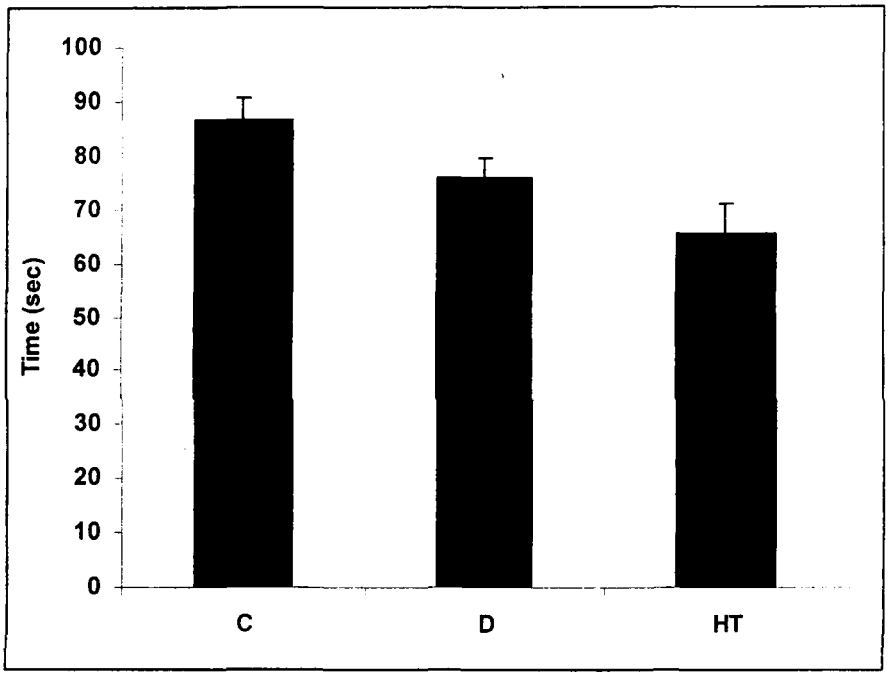


Fig.1.3: sdLDL oxidation time of diabetic and hypertensive subjects.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM $CuCl_2$ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

1.4: PON-1 activity

PON-1 is an HDL-associated enzyme that has been identified as an important determinant of the capacity of HDL to prevent oxidative modification to LDL. Aviram et al. (1998) showed that the esterolytic activity of PON-1 in serum HDL correlated inversely with the susceptibility of HDL to oxidation. The activity of this enzyme has also been reported to be decreased in cardiovascular diseases (Navab et al., 1997 and McElveen et al., 1986) and in diabetes mellitus (Mackness et al., 1998 and Sakai et al., 1998). A few scientists have observed reduced levels of serum PON-1 in the patients with metabolic syndrome (Garin et al., 2005). In light of these observations, serum PON-1 levels were measured in this study and the results obtained were quite similar. Fig.1.4 shows that the PON-1 activity was significantly reduced in case of hypertensive (HT) and diabetic subjects (D). However, no such diminution was seen in the control subjects (C) without diabetes or hypertension. This supports the notion that qualitative changes to the LDL and HDL profiles in metabolic syndrome is accompanied by a reduction in the capacity of one of the main antioxidant activities associated with HDL (Garin et al., 2005). This could be a contributory factor to the increased incidence and severity of CVD observed in metabolic syndrome affected populations.

1.5: CRP levels

Recently several studies have drawn attention to the finding of elevated levels of CRP, a sensitive marker of inflammation, in subjects of metabolic syndrome or in association with its components (Haverkate et al., 1997; Koenig et al., 1999; Mendall et al., 1996). Elevated levels of CRP were also found in type 2 diabetic patients with features of metabolic syndrome (Pickup et al., 1997). Similar results were obtained following the estimation of serum CRP levels of the subjects involved in this study.

Fig.1.5 represents the CRP levels in control, diabetic and hypertensive subjects. It is clearly depicted that CRP levels of both diabetic (D) and hypertensive subjects (HT) were greater than that of control subjects (C). The importance of this finding stems from the evidence, that elevation in CRP level is associated with an increased risk of metabolic syndrome and cardiovascular events (Florez et al., 2006). These results strongly suggest that CRP levels might provide an additional measure for identifying subjects for these co-morbidities and who therefore, would benefit from preventive interventions.

1.6: TNF-α levels

Metabolic syndrome accompanies nearly all patients who become obese, yet the relationship between adiposity and insulin sensitivity is not clear. A possible aetiology for insulin resistance in obesity is the production of the cytokine, TNF-α. Recent human studies have demonstrated elevated TNF-α expression in the adipose tissue of obese subjects, and its decreased expression following weight loss (Hotamisligil et al., 1995 and Kern et al., 1995). Nonetheless, the present study reveals higher levels of TNF-α at the onset of diabetes and hypertension (fig.1.6). It is clearly depicted that TNF-α levels were always greater in case of hypertensive subjects (HT), followed by the diabetic (D) and control subjects (C). Previously reported elevation in the waist circumference of both diabetic and hypertensive subjects might provide a coherent explanation for such a change in TNF-α levels. These results are consistent to the earlier reports demonstrating a strong relationship between TNF-α and metabolic syndrome (Bertin et al., 2000). These findings strongly recommend the estimation of TNF-α in prediction of metabolic syndrome.

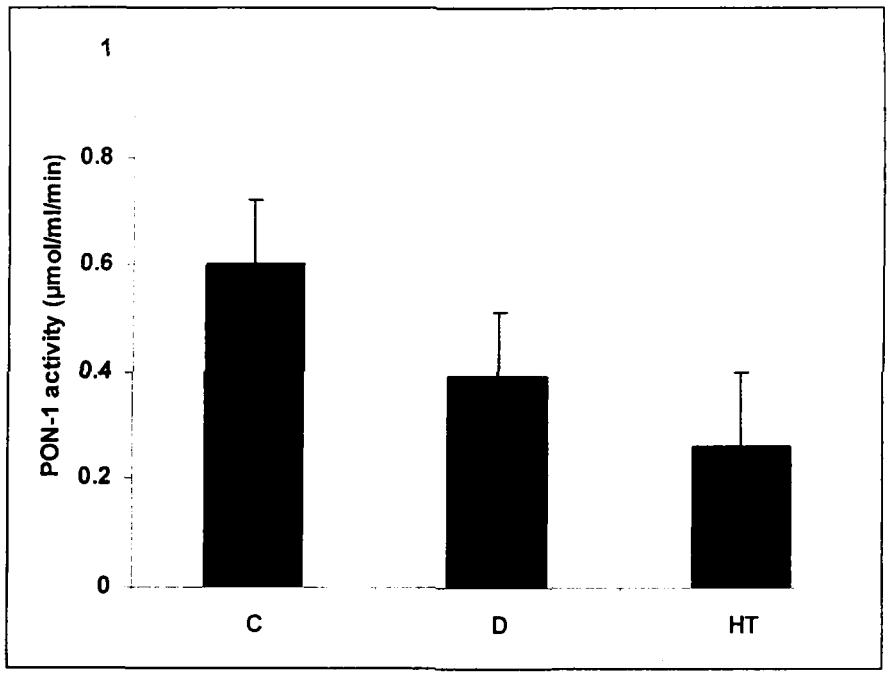


Fig.1.4: PON-1 activity of diabetic and hypertensive subjects.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM $CaCl_2$ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

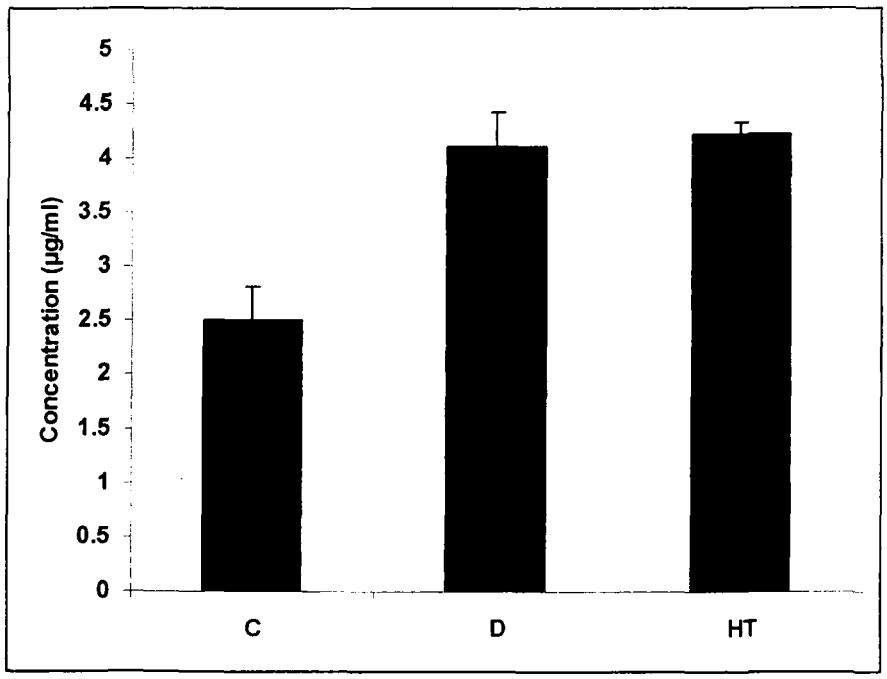


Fig.1.5: CRP levels of diabetic and hypertensive subjects.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

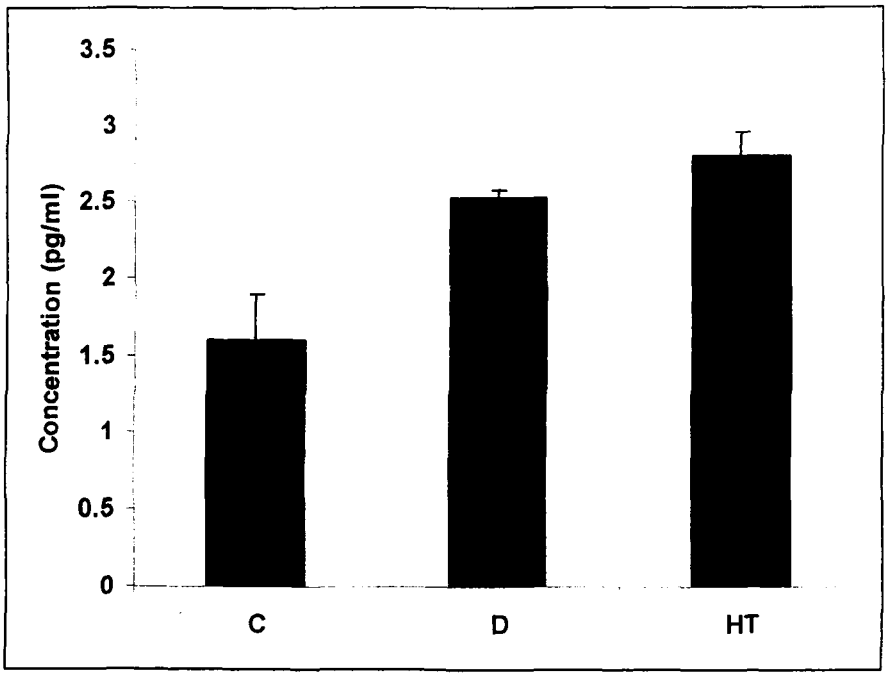


Fig.1.6: TNF-α levels of diabetic and hypertensive subjects.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

1.7: Apo-B levels

Apo-B is the primary apolipoprotein of low density lipoproteins (LDL), which is responsible for carrying cholesterol to tissues (Chumakova et al., 2005). It has recently been found to be associated with metabolic syndrome and a few studies have also revealed the association of Apo-B levels of diabetic persons with metabolic syndrome (Relimpio et al., 2002; Onat et al., 2007). In another study, including 77 non-diabetic postmenopausal overweight and obese women, apo-B was found to be the primary predictor when compared with various inflammatory markers (like IL-6, CRP, TNF-α and others) among a variety of risk parameters such as adiposity, blood pressure, insulin resistance, TGs, etc (Faraj et al., 2006). It was suggested that high apo-B would be associated with increased risk of developing coronary heart disease and diabetes. These findings stimulated the verification of apo-B levels in the diabetic and hypertensive subjects included in present study. This was done to predict the risk of metabolic syndrome. Results presented in fig.1.7 perpetuate that the apo-B levels were high in both diabetic (D) and hypertensive subjects (HT). The control subjects (C) having neither diabetes nor hypertension, showed a noticeably low level for apo-B. This result further adds to the hypothesis that apo-B probably has a potential role in the development of metabolic syndrome.

1.8: Correlation between NCEP/ATP III parameters and observed parameters of metabolic syndrome

The NCEP/ATP III guidelines to propose metabolic syndrome state that a person must have three of the following five abnormalities: abdominal adiposity, hypertension, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol, and hyperglycemia. Eventually, the prevalence of metabolic syndrome in diabetic and hypertensive subjects included in this study was also estimated on the basis of same criteria. In addition, the role

of sdLDL oxidation and serum paraoxonase (PON-1) activity in prediction of metabolic syndrome has also been recognized recently (Nishtha et al., 2008a). Hence, it may be assumed that there exist some relationship between the parameters incorporated in NCEP/ATP III guidelines and the other parameters being evaluated in this study. Fig.1.8 represents a correlation between the NCEP/ATP III components and the other observed factors namely CRP, TNF-α and apo-B levels together with sdLDL oxidation time and PON-1 activity measured in each group. It was witnessed that a significantly positive correlation was found between the levels of CRP, TNF-α, apo-B and NCEP/ATP III parameters. This implies elevation in all these parameters as metabolic syndrome progresses. On the other hand, a significantly negative correlation was deduced between sdLDL oxidation time as well as PON-1 activity with respect to NCEP/ATP III parameters. This reveals the existence of an inverse relationship of these parameters to metabolic syndrome. Thus, explains their declination as metabolic syndrome progresses.

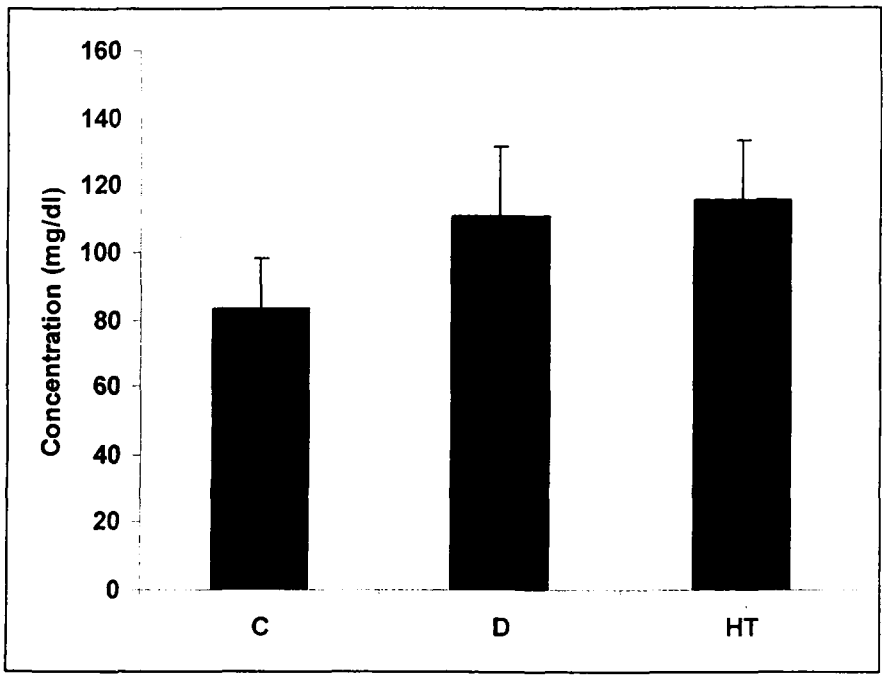


Fig.1.7: Apo-B levels of diabetic and hypertensive subjects.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group

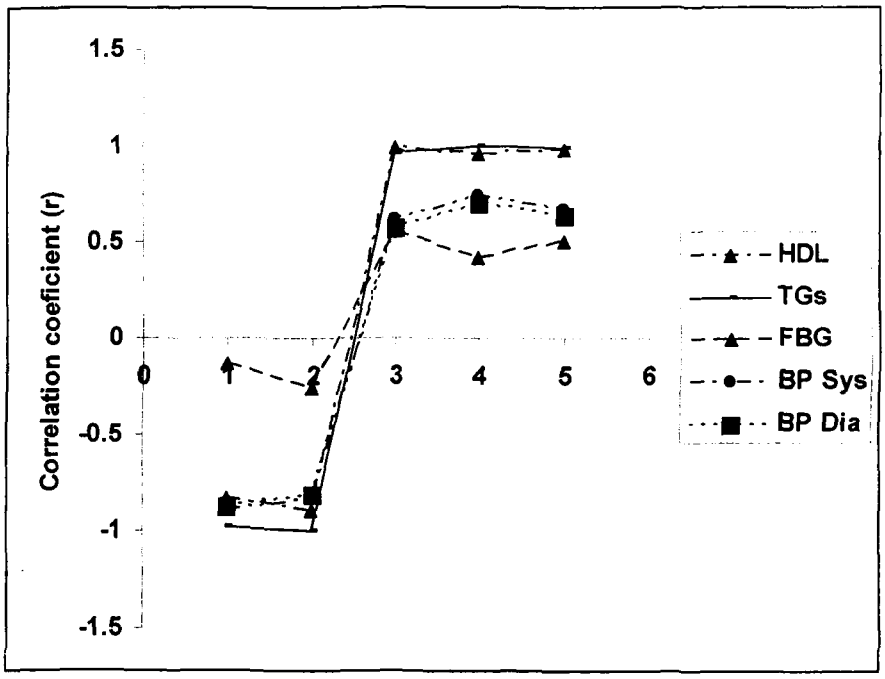


Fig.1.8: Correlation between NCEP/ATP III parameters and observed parameters of metabolic syndrome in diabetic and hypertensive subjects.

Pearson's correlation analysis was used for determination of relationship between NCEP/ATP III parameters and observed parameters of diabetic and hypertensive subjects. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to get a positive or negative relationship.

1 sdLDL oxidation time
2 PON-1 activity
3 CRP concentration
4 TNF-α concentration
5 Apo-B concentration

Chapter 2
Treatment of Metabolic Syndrome (Human Studies)

Unit 2 (A)

Treatment of Metabolic Syndrome in Diabetic Subjects

Metformin {(1-(diaminomethylidene)-3,3-dimethylguanidine) is an inexpensive compound with documented glucose-lowering effect in both obese and non-obese subjects having type 2 diabetes (Consoli et al 2004; Donnelly et al 2006). The reduction of glycemic levels upon supplementation with metformin is done primarily by inhibiting hepatic glucose output (Leverve et al 2003). The beneficial effects of metformin on parameters of the metabolic syndrome in clinical diabetes were confirmed by the UK Prospective Study Group (UKPDS, 1998a) and it was the only drug in this mega-trial that significantly reduced cardiovascular events.

Insulin analogs have also been developed by modifying the amino acid sequence of the insulin molecule. Insulin is available in rapid, short, intermediate, and long-acting types that may be injected separately or mixed in the same syringe. Rapid-acting insulin analogs (insulin lispro and insulin aspart) are available, and other analogs are in development. Regular is short-acting insulin. Intermediate-acting insulins include lente and NPH. Ultralente and insulin glargine are long-acting insulins. Insulin preparations with a predetermined proportion of intermediate-acting insulin mixed with short or rapid-acting insulin (for example, 70% NPH/30% regular, 50% NPH/50% regular, and 75% NPH/25% insulin lispro) are also available (American Diabetes Association, 2002).

The present study investigates whether a monotherapy with metformin or insulin (mixture of intermediate and short acting insulin) has any beneficial role or not in metabolic syndrome and DNA damage. The parameters being analyzed herein follow the guidelines of NCEP/ATP III and include the determination of fasting blood glucose, HDL, and triglyceride levels in all the subjects involved. In addition to this, sdLDL oxidation time, serum paraoxonase (PON-1) activity, concentration of inflammatory markers (CRP and TNF-α) as well as apo-B and lipid peroxides levels were calculated as earlier reports from this laboratory have published their role in metabolic syndrome (Nishtha et al., 2008b). Previous studies have also demonstrated

an association between increased DNA damage and diabetes (Collins et al., 1998). In light of these observations, the extent of DNA damage in diabetic subjects as well as the effect of metformin or insulin monotherapy against this damage was also monitored.

2A.1: Anthropometric observations

Improved glycemic control in diabetes has, to date, not shown any significant reduction in CVD. In fact, cardiovascular events increased in the Veterans Affairs Cooperative Study on Glycemic Control and Complications in Type II Diabetes (VACSDM), and the decrease in cardiovascular events in the United Kingdom Prospective Diabetes Study (UKPDS) was not statistically significant. In contrast, insulin given acutely as an intravenous infusion has shown a favorable effect on several risk factors of CVD (Chaudhuri et al., 2004).

In an effort to identify the effect of glycemic control in reducing metabolic syndrome, a detailed analysis was performed on diabetic subjects receiving metformin monotherapy (500 mg, orally administered once daily), and diabetic subjects receiving insulin monotherapy (10 I.U. injected sub-cutaneously, twice a day). Diabetic subjects receiving either of the two monotherapies, as already prescribed by their physicians were on the regular treatment for past 5 months and not receiving any other medication for hyperglycemia. Measurement of all the components of metabolic syndrome, established in previous section was also performed in this study. Non-diabetic controls (C) as well as the diabetic subjects who were not receiving any hypoglycemic drug (D+ND) were also chosen in addition to the metformin or insulin supplemented subjects and their anthropometric observations are listed in table.2A.1. It is depicted that the diabetic subjects dependent on insulin (D+I) were from a comparatively elder age group. Waist circumference of all the categories remained almost same except the control

group (C), where it was found to be significantly low. However, a major difference was noticed in the blood glucose levels of all subjects. It is witnessed from the table that FBG levels reduced significantly in insulin treated subjects followed by metformin treated subjects among the diabetic population. This is coherent to the previous reports suggesting reduction in blood glucose in overweight diabetic subjects upon administration of metformin as well as insulin (Bailey and Turner, 1966; Inzucchi et al., 1998; Phillips et al., 2003).

2A.2: HDL levels

Previous studies of diabetes treatment were limited to the glycemic control only. Modern therapies however also imply the role played by blood lipids in the progression of diabetes (deFronzo and Goodman, 1995; Campbell, 2000). In the present study, HDL levels of all subjects were measured to judge their relevance to metabolic syndrome. Fig.2A.2 represents an insignificant variation in HDL levels of the four categories. Diabetic subjects receiving insulin or metformin monotherapy (D+I, D+M) however, reported slightly elevated amounts of HDL. Thus indicating a plausible role of insulin as well as oral hypoglycemics in improvement of lipid profile, which is a major decisive factor of metabolic syndrome. This result is consistent to the findings of Fonseca et al. (2006) highlighting increased HDL levels upon insulin and metformin administration.

2A.3: TGs levels

The metabolic syndrome is frequently associated with low HDL and hypertriglyceridemia (Steiner, 2004). Poorly controlled diabetes causes hypertriglyceridemia and therefore, drug treatment should be effective not only for the lipid disorder but also, in the best case, to improve insulin resistance and glucose tolerance (Meyers and Kashyap, 2004). It was

observed that the metformin treated group (D+M) demonstrated remarkably decreased concentration of TGs (fig.2A.3) followed by the levels in insulin treated group (D+I), and no drug group (D+ND). Thus, suggesting the role of oral hypoglycemics in reducing TGs levels and bringing it near normal levels as shown in the control group (C). This effect of metformin adds up to the reason why oral hypoglycemics are the drugs of first choice in diabetic subjects who also suffer from metabolic syndrome.

2A.4: Lipid peroxidation

It has recently been reported from this laboratory that concentration of lipid peroxides in oxidized sdLDL is an important marker of metabolic syndrome and oxidative stress (Nishtha et al., 2008b). Thus, the sdLDL was isolated, oxidized by cupric chloride and lipid peroxides were measured in the present study groups. Fig.2A.4 shows the lowest concentration of lipid peroxides in metformin treated group (D+M). The diabetic group amid insulin administration (D+I) also witnessed a noticeable reduction when compared to the group receiving no drug at all (D+ND). This suggests that the maximum lipid peroxidation occurred in diabetic group receiving no treatment and the lowest in non-diabetic control (C) group. This result also indicates that the production of lipid peroxides is increased upon initiation of diabetes which is abridged by the supplementation of either oral hypoglycemics or insulin. Similar studies involving both metformin and insulin as therapeutic targets for diabetes have also reported decreased lipid peroxidation (Fonseca et al., 2006). Thus, the result generates a necessity for the provision of adequate basal insulin or in other words, proper glycemic control to lower the lipid peroxidation and presumably, oxidative stress leading to metabolic syndrome.

Parameters	C (n=28)	D + ND (n=30)	D + I (n=35)	D + M (n=35)
Males (n) Females (n)	12 16	25 5	28 7	20 15
Age (Yrs)	36 ± 3.1	46 ± 8.1	52 ± 9.3	48 ± 8.7
WC (cm)	79 ± 1.8	102 ± 1.3	110 ± 2.3	105 ± 2.8
FBG (mg/dl)	96 ± 1.5	170 ± 2.3	120 ± 1.7	136 ± 1.5

Table.2A.1: Anthropometric observations in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The blood glucose level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 120 mg/dl. The waist circumference (WC) for each subject was measured by the usual procedures as described in 'methods'. Results are expressed as mean ± S.D.

C Control
D + ND Diabetic + No Drug
D + I Diabetic + Insulin
D + M Diabetic + Metformin

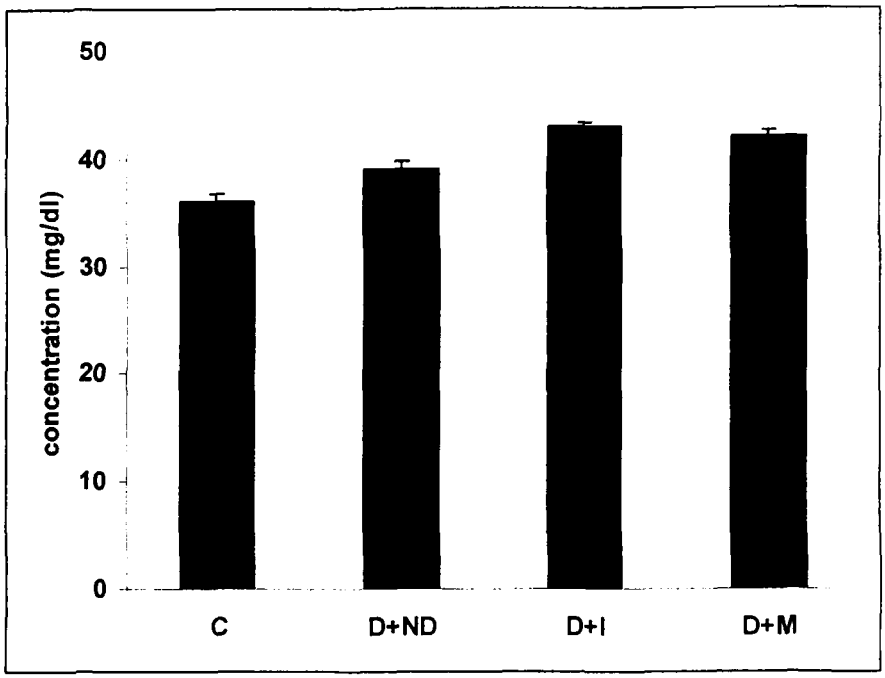


Fig.2A.2: HDL levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

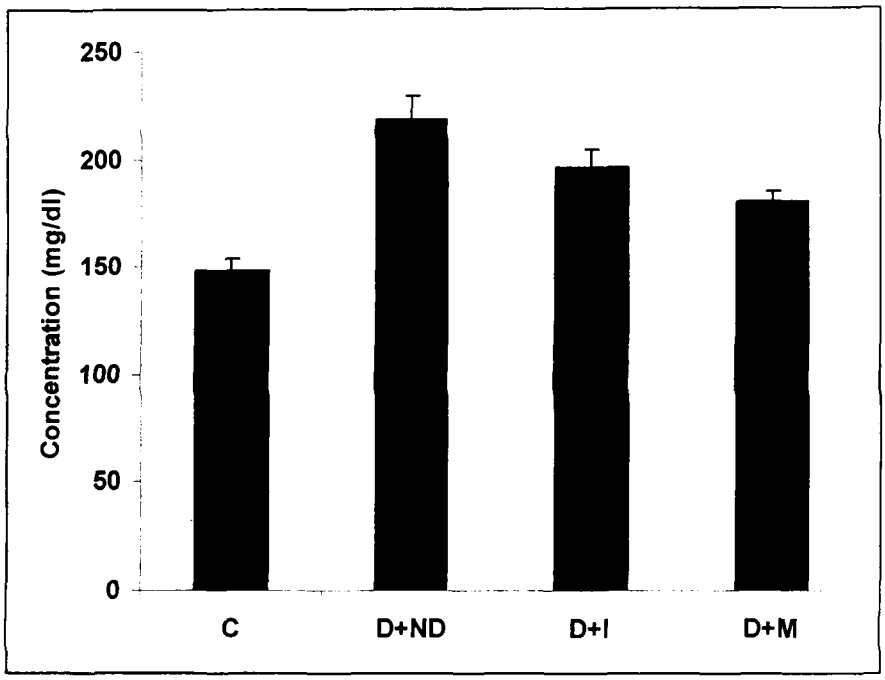
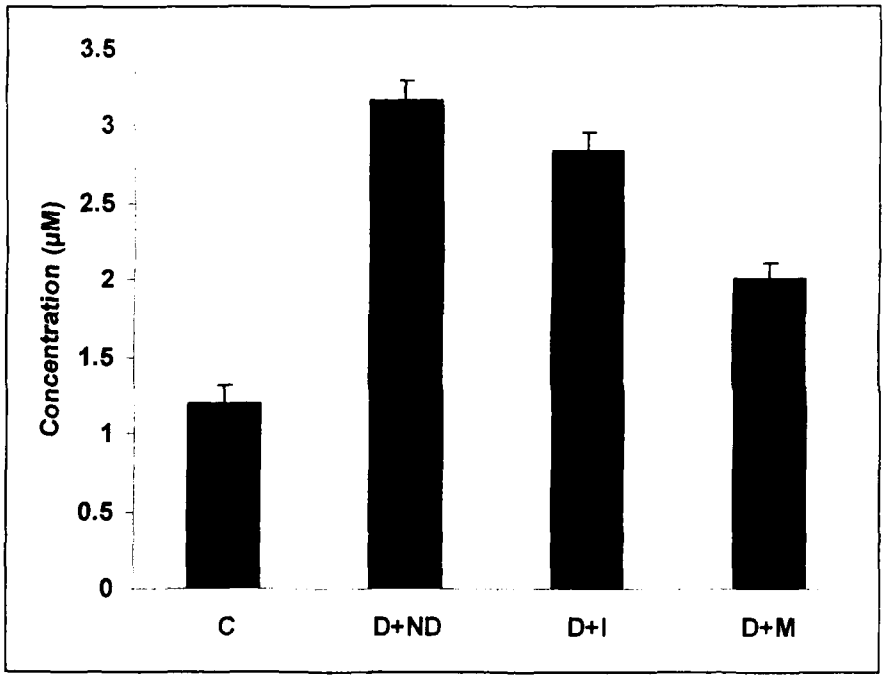


Fig.2A.3: TGs levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin



2A.4: Lipid peroxidation in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

2A.5: PON-1 activity

Previous results from the same study have revealed insignificantly elevated concentration of HDL in diabetic subjects after insulin or metformin monotherapy. As serum paraoxonase (PON-1) is an HDL-associated enzyme that protects both HDL and LDL from oxidation, a curiosity was raised to determine the content of this enzyme also. The results obtained after performing PON-1 assay in all the four categories came out to be analogous to what was expected. Fig.2A.5 reveals the rise in activity of this enzyme in case of diabetic subjects treated with metformin (D+M) followed by those receiving insulin (D+I). The PON-1 activity of diabetic subjects receiving no medication (D+ND) showed significantly low-levels whereas; the non-diabetic control group (C) showed the highest levels of enzyme activity. These results were comprehensible to the earlier proposed hypothesis stating that metformin possess antioxidant properties and significantly lowers insulin resistance (Faure et al., 1999).

2A.6: CRP and TNF-α levels

Both CRP and TNF-α are the markers of inflammation that have been associated with diabetes, metabolic syndrome and CVD (Tracy et al., 1997). There are different views among scientists, regarding the effect of oral hypoglycemics as well as insulin analogues on the CRP levels of diabetic patients. Yudkin et al. (1999) hypothesized the association of high CRP levels and oxidative stress. Further, Dandona et al. (2002) established the anti-inflammatory properties of intravenous insulin. The results explained in fig.2A.6(a) indicate high CRP levels in the diabetic subjects receiving no drug (D+ND) as compared to the non-diabetic control subjects (C). However, an insignificant reduction in CRP levels was also noticed in diabetic subjects after insulin or metformin monotherapy (D+I, D+M).

The implication of TNF-α in the pathogenesis of metabolic syndrome has been attributed to the over-expression of TNF-α gene in the adipose

tissue of obese humans (Hotamisligil et al., 1995; Kern et al., 1995) and rodents (Hotamisligil, 1993; Hotamisligil et al., 1996). Moreover, certain studies have also reported an association of increased TNF-α with insulin resistance (Hotamisligil, 1993; Ruan and Lodish, 2003). Thus, the increment in the levels of TNF-α in diabetic subjects (D+ND) as displayed in fig.2A.6(b), may account for the development of metabolic syndrome. Despite this risk, a slender, non-significant attenuation of TNF-α levels was also observed after administration of insulin or metformin alone (D+I, D+M).

2A.7: Oxidation of sdLDL and Apo-B levels

Circulating levels of apolipoproteins reflect the number of, rather than the cholesterol concentration of, lipoprotein particles. Specifically, the level of apo-B reflects the number of triglyceride-rich VLDL particles and the number of LDL particles. It thus gives more credence to the number of sdLDL particles than the more regular measurement of LDL cholesterol (Lind et al., 2006). Assessment of qualitative changes in sdLDL is therefore more significant in metabolic syndrome because of its ability to get oxidized easily (Scheffer et al., 2003). Such an important role of Apo-B and sdLDL in determination of metabolic syndrome has provided grounds for their estimation in the present study. Therefore, the time taken for oxidation of sdLDL, isolated from different groups was monitored. Surprisingly, it was found that oxidation of sdLDL from diabetic subjects (D+ND) completed in minimum time when compared to the one observed for non-diabetic control subjects (C) and diabetic subjects treated with metformin (D+M) or insulin (D+I) [fig.2A.7(a)]. Increase in the time required for oxidation of sdLDL in diabetic subjects receiving metformin depicts qualitative changes in sdLDL particles of these subjects, and thus reducing their chances of developing atherosclerosis and metabolic syndrome.

Similarly, fig.2A.7(b) reveals the effect of metformin or insulin supplementation on the apo-B levels of diabetic subjects. It is quite

remarkable that apo-B levels significantly decreased when diabetic subjects were treated with metformin (D+M). A lesser reduction was also observed in the insulin treated subjects (D+I) as compared to those receiving no drug (D+ND). Induction of these changes in sdLDL and apo-B by metformin administration may be one of the mechanisms of the anti-atherogenic effect of such oral hypoglycemics.

2 A.8: Oxidative stress marker MDA

There is growing evidence that excess generation of highly reactive free radicals, largely due to hyperglycemia, causes oxidative stress, which further exacerbates the development and progression of diabetes and its complications (Johansen et al., 2005). Also it has been reported that uncontrolled hyperglycemia was associated with elevated levels of malondialdehyde (MDA), an important marker of oxidative stress, which may get reduced with the supplementation of numerous antioxidant compounds (Vega-Lopez et al., 2004). Therefore it was of interest to measure MDA levels in serum of the diabetic subjects receiving either metformin or insulin monotherapy. Fig.2A.8 demonstrates elevated MDA levels in the diabetic group receiving no treatment (D+ND) denoting increased oxidative stress. On the other hand, metformin and insulin treated groups (D+M, D+I) showed remarkably decreased MDA levels; metformin revealing enhanced antioxidant effect as compared to that of insulin. Hence, it may be affirmed that in diabetic condition, free radicals are elevated and metformin or insulin treatment attenuated these changes.

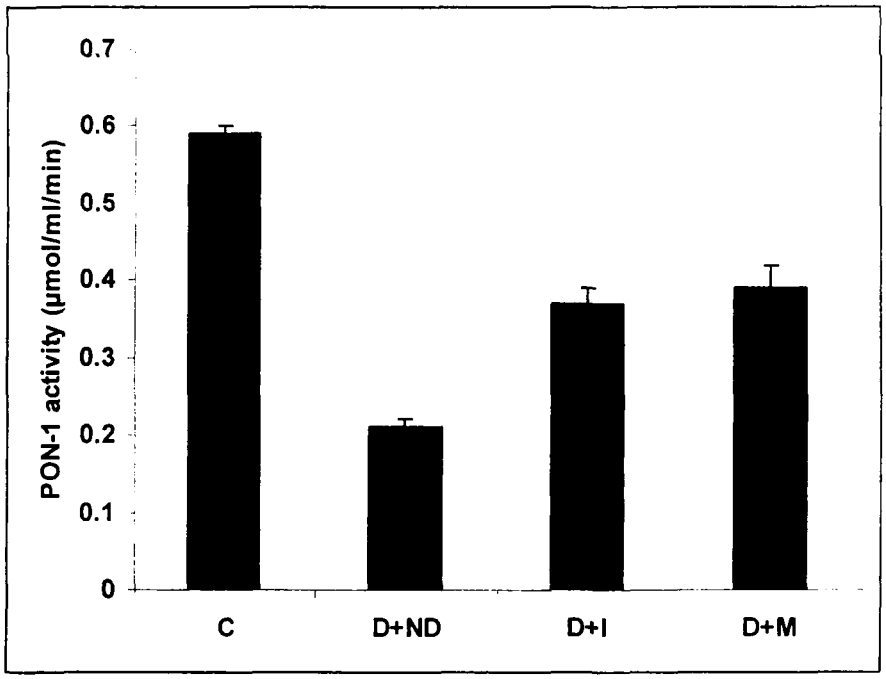


Fig.2A.5: PON-1 activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM $CaCl_2$ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

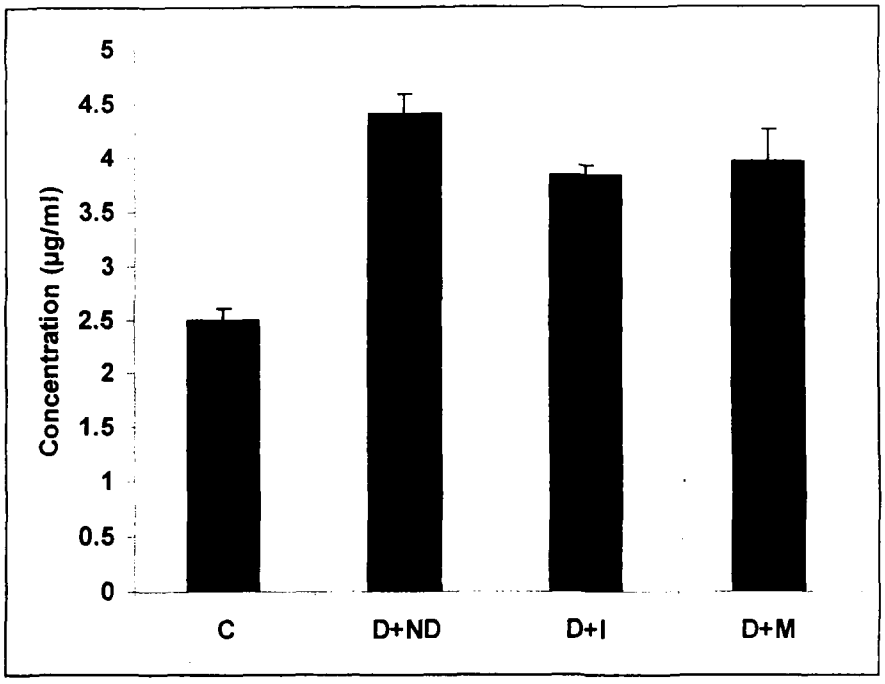


Fig.2A.6(a): CRP levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

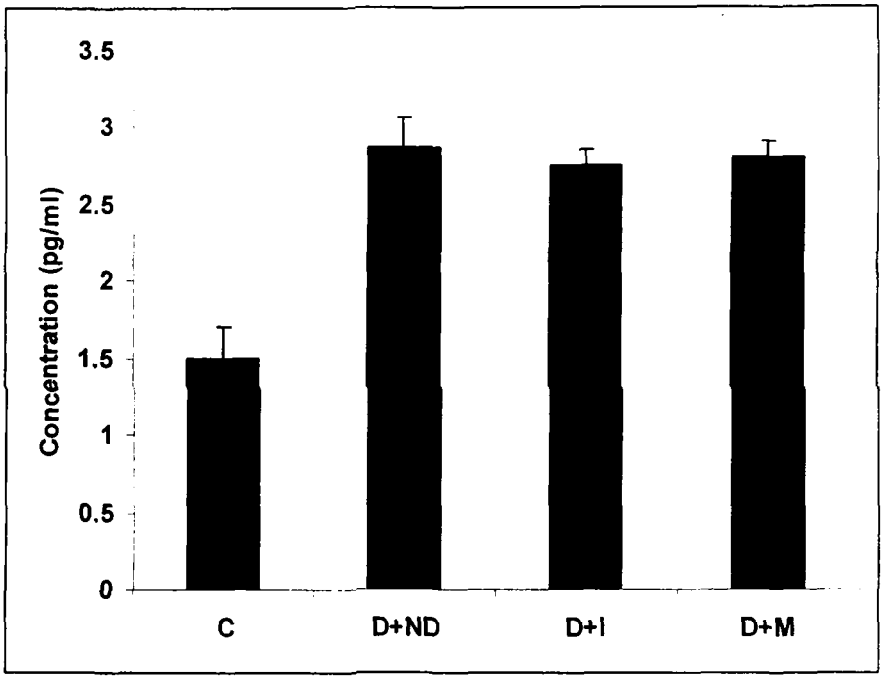


Fig.2A.6(b): TNF-α levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

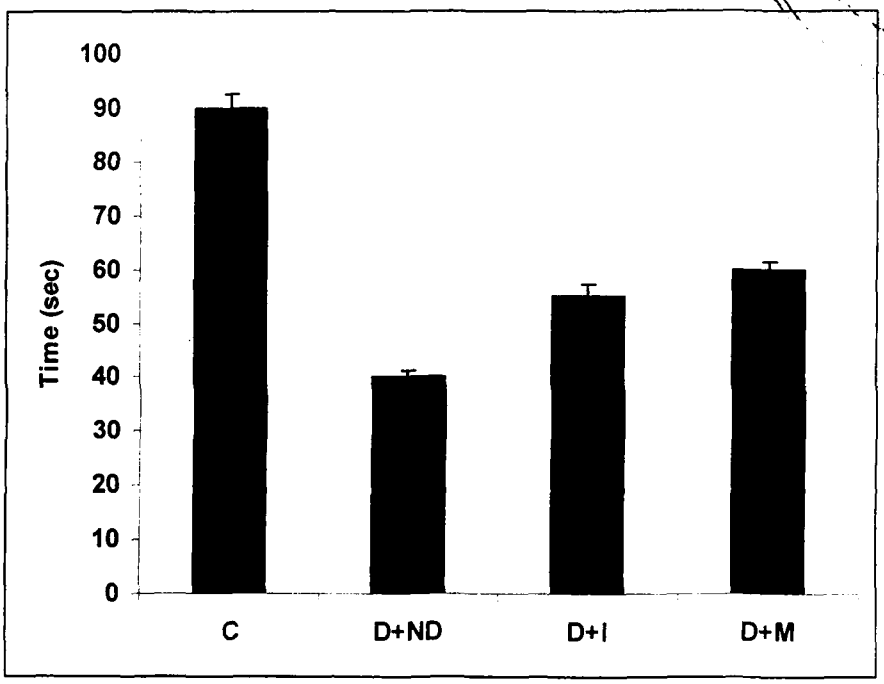


Fig.2A.7(a): sdLDL oxidation time of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM $CuCl_2$ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

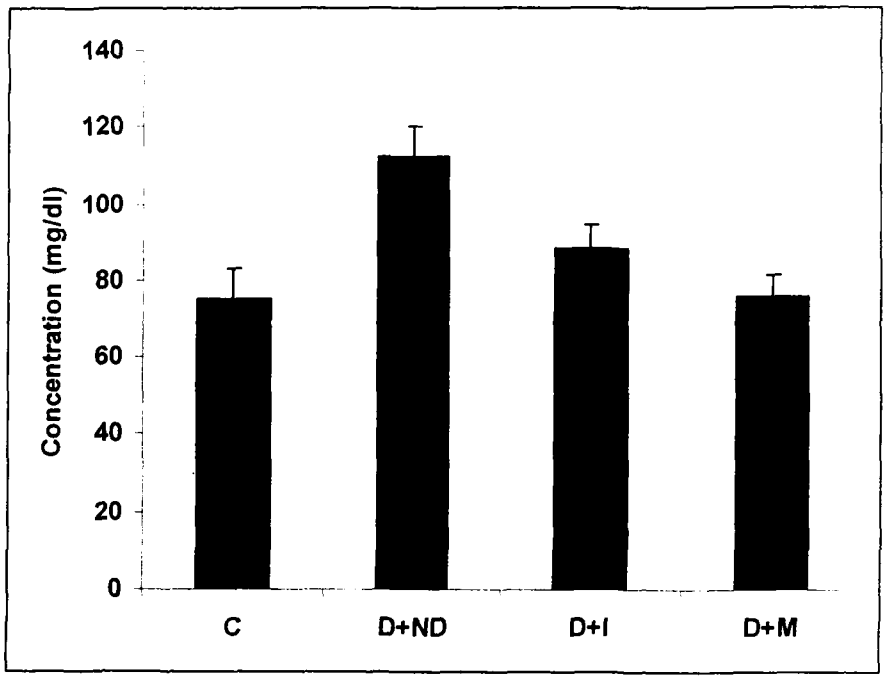


Fig.2A.7(b): Apo-B levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

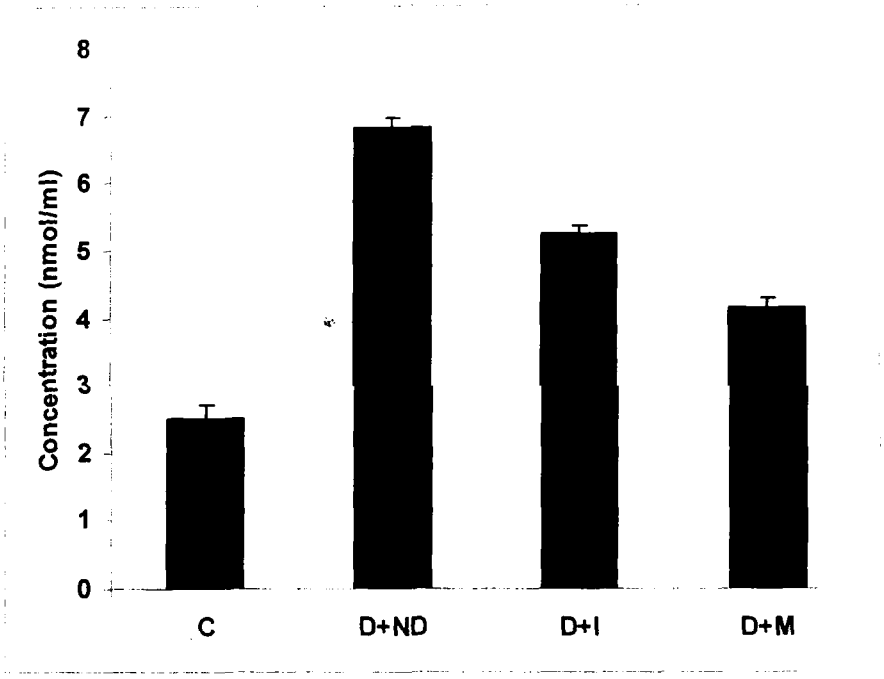


Fig. 2A.8: MDA levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in 'methods'. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

2A.9: SOD and Catalase activity

Maritim and colleagues recently reviewed in detail that diabetes has multiple effects on the protein levels and activity of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which further augment oxidative stress by causing a suppressed defense response (Maritim et al., 2003). SOD immediately converts superoxide radical to hydrogen peroxide, which is then detoxified to water either by catalase in the lysosomes or by glutathione peroxidase in the mitochondria. A plethora of studies have been performed to determine the activity of these enzymes upon induction of diabetes and after supplementation with metformin or insulin (Ramanathan et al., 1999; Pavlovic et al., 2000; Kocic et al., 2007). All these studies have emphasized the role of these hypoglycemics in ameliorating oxidative stress and implicate that such a treatment significantly increased the level of antioxidant enzymes and reduced the amount of lipid peroxidation. The results indicated in fig.2A.9(a,b) also witnessed similar outcomes and elucidate that the enzyme activity of SOD as well as catalase was highest in the non-diabetic control group (C) and decreased significantly in diabetic subjects receiving no treatment (D+ND). It is interesting to note that though both metformin and insulin monotherapy succeeded in augmentation of SOD and catalase activity, the results of treatment with metformin (D+M) showed much significant values as compared to that of treatment with insulin (D+I). This investigation suggests that the application of metformin monotherapy in diabetic subjects improves the antioxidative enzyme status considerably and thus adds to an important preventive measure for oxidative stress.

2A.10: DNA damage

Insulin dependent diabetes mellitus is associated with increased oxidative stress *in vivo* and under conditions of oxidative stress; damage to cellular biomolecules (lipids, proteins, carbohydrates, and DNA) can occur (Hannon-Fletcher et al., 2000). Such damage in the DNA of diabetic subjects can be

measured by a technique known as comet assay. The comet assay, can potentially measure DNA lesions in any organ or tissue even in the absence of mitotic activity. Cells are examined using fluorescent microscope. Cells look like comets with a bright fluorescent head and tail, the length and intensity of which are related to the number of strand breaks (Dobrzynska, 2005).

Traditionally, peripheral blood lymphocytes have been preferred because they are regarded as sentinel cells being early warning signals for adverse health effects (Tice et al., 2000). The assay involves treatment of small number of cells layered on glass slides and sandwiched between layers of agarose. Furthermore, agarose embedded cells are lysed to generate a nucleoid body composed of nuclear DNA stripped of some attached protein and its histones. These nucleoid bodies are then subjected to limited electrophoresis that cause the damaged DNA to migrate away from the undamaged DNA forming a "comet", in which the 'head' of the comet is the undamaged DNA and the 'tail' the damaged DNA. Therefore, the more damaged a cell's DNA is, the greater the amount of DNA in the tail (often measured as olive tail movement, OTM or tail length) (Almeida et al., 2006). The size of the comet and the distribution of fluorescence within it are correlated quantitatively with the frequency of DNA breaks (Fairbairn et al., 1995).

Several studies have shown increases in the level of DNA damage in subjects with poor glycemic control (Collins et al., 1998 and Lorenzi et al., 1987), and no significant changes in subjects with good glycemic control (Anderson et al., 1998). To verify the extent of DNA damage in untreated diabetic subjects and those receiving a hypoglycemic treatment, fresh lymphocytes were isolated from the blood samples of each subject and the procedures of comet assay were followed. Interestingly, this study also presented similar results when DNA damage was assessed for non-diabetic control subjects (C), diabetic subjects receiving no medication (D+ND), and

diabetic subjects treated with oral metformin (D+M) or sub-cutaneously supplied insulin (D+I). Fig.2A.10 represents the extent of DNA damage in all the above stated categories and witnesses no significant alteration in tail-length of subjects from any of the three diabetic groups. DNA damage in the non-diabetic control subjects (C) was however lesser than any other category stated. This result implies that supplementation of oral hypoglycemics or even insulin does not provide any significant protection to the DNA damage.

2A.11: Correlation between NCEP/ATP III parameters and observed parameters

Present study engrosses the determination of glycemic control and its effect on metabolic syndrome. The NCEP/ATP III components (abdominal adiposity, hypertension, hypertriglyceridemia, low HDL, and hyperglycemia) as well as the parameters observed in present study (CRP, TNF-α and apo-B levels together with sdLDL oxidation time, lipid peroxidation, and PON-1 activity) have been shown to be involved in metabolic syndrome in the earlier chapter. Several reports have also revealed an increased DNA damage in diabetes (Collins et al., 1998; Lorenzi et al., 1987; Anderson et al., 1998). In an effort to establish a relationship between these factors and NCEP/ATP III parameters in diabetic subjects with the administration of insulin or metformin, Pearson's correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.2A.11 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs and FBG, (ii) lipid peroxidation and HDL, (iii) CRP and HDL, (iv) sdLDL oxidation time and levels of TGs and FBG, (v) TNF-α and HDL, (vi) apo-B and HDL, (vii) DNA damage and HDL. Thus it adds to an important observation depicting the reduction in lipid peroxidation, and levels of CRP, apo-B and TNF-α together with the DNA damage upon elevation in HDL concentration followed by insulin or metformin administration. Similarly, the decrease in TGs and FBG

concentration as a result of the treatment with these hypoglycemics was found to be responsible for increased PON-1 activity and the time required for oxidation of sdLDL. This implies that regulation of FBG, TGs and HDL levels after treatment with hypoglycemics, may play a crucial role in preventing the development of metabolic syndrome and other risk factors associated with it.

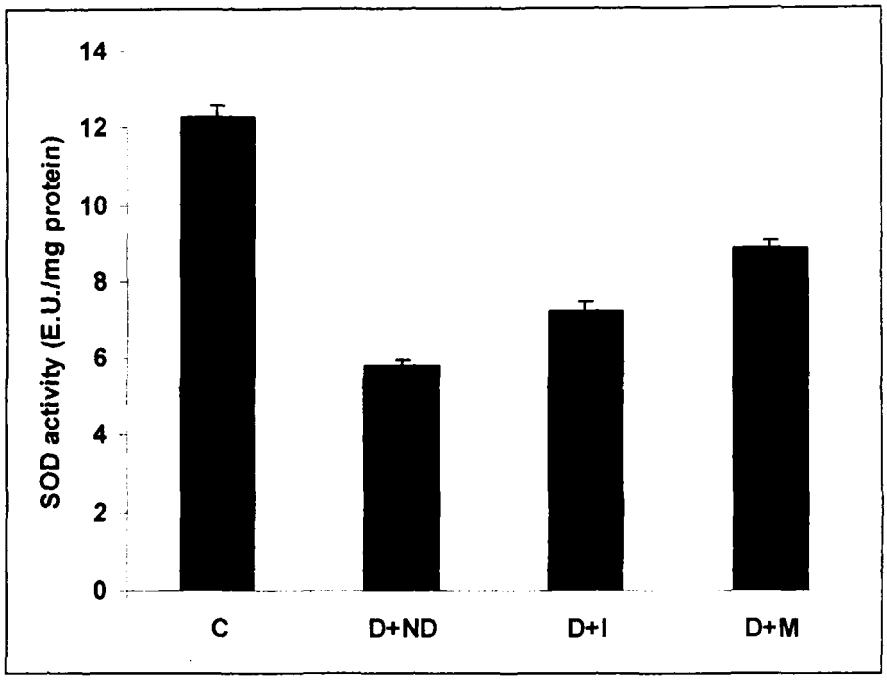


Fig.2A.9(a): SOD activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The assay medium in a final volume of 3 ml consisted of 50 µl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit (E.U.) is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

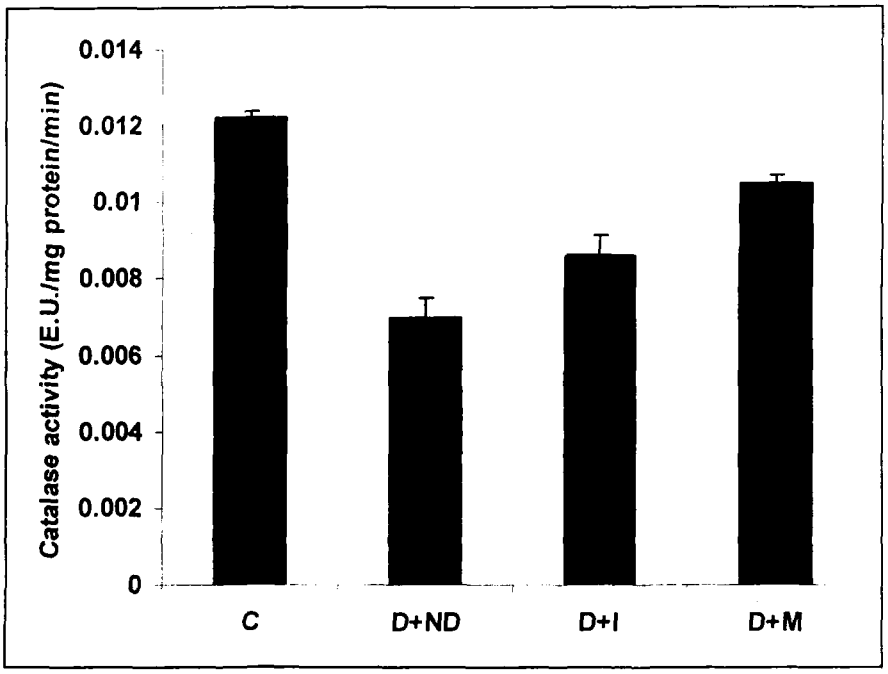


Fig.2A.9(b): Catalase activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H_2O_2). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml H_2O_2 (30 mM) and 50 µl sample. One enzyme unit (E.U.) is defined as the amount of enzyme decomposing 1 µM H_2O_2 per min at 25°C. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

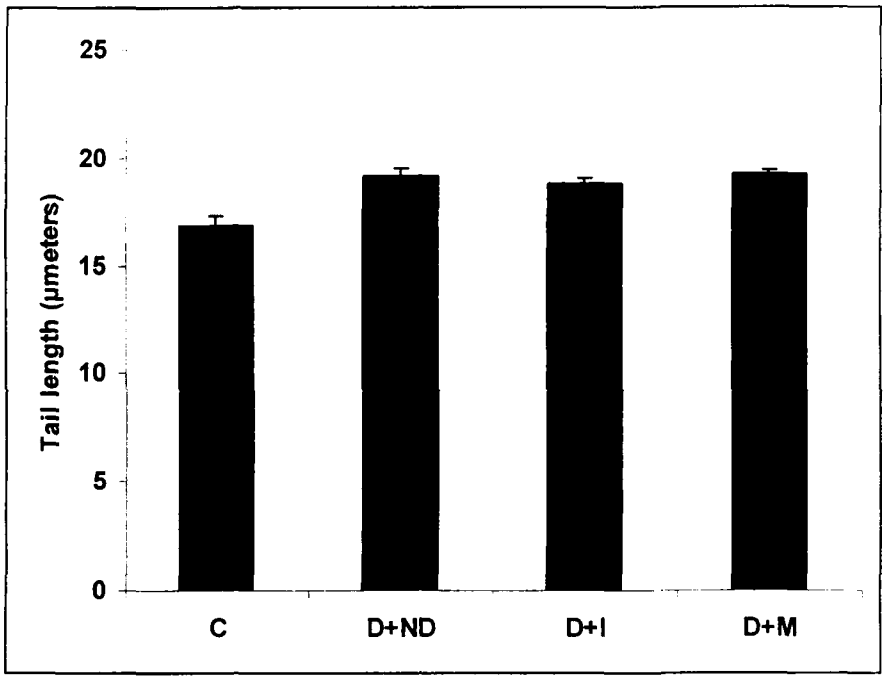


Fig.2A.10: DNA damage in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

DNA breakage in the lymphocytes isolated from each subject was measured by comet assay proposed by Singh et al. (1988) as described in 'methods'. Comet tail length (µmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

C	Control
D + ND	Diabetic + No Drug
D + I	Diabetic + Insulin
D + M	Diabetic + Metformin

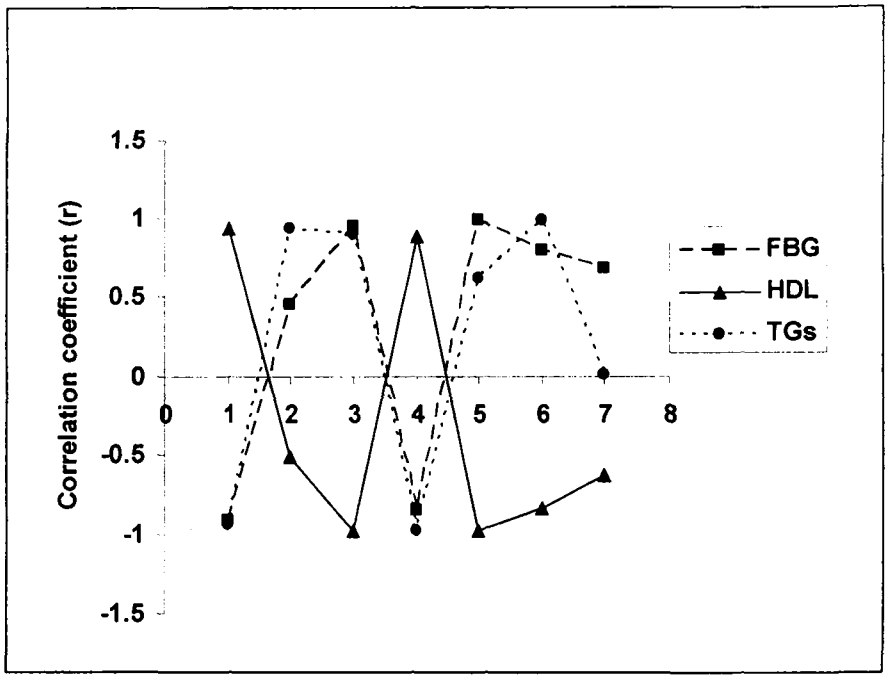


Fig.2A.11: Correlation between NCEP/ATP III parameters and observed parameters with insulin or metformin monotherapy.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed in diabetic subjects with the administration of insulin or metformin. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1 PON-1 activity
2 Lipid peroxidation
3 CRP concentration
4 sdLDL oxidation time
5 TNF-a concentration
6 Apo-B concentration
7 DNA damage

Unit 2 (B)

Treatment of Metabolic Syndrome in Hypertensive Subjects

Angiotensin II [formed from angiotensin I in a reaction catalyzed by angiotensin converting enzyme (ACE, kininase II)] is a potent vasoconstrictor, the primary vasoactive hormone of the rennin-angiotensin system and an important component in the pathophysiology of hypertension. It also stimulates aldosterone secretion by the adrenal cortex. Losartan and its principal active metabolite block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the angiotensin I receptor found in many tissues (vascular smooth muscle, adrenal gland) and thus called as Angiotensin receptor blocker (ARB) or Angiotensin receptor antagonist (ARA) (Vitale et al., 2005).

Ramipril, on the other hand inhibit ACE in human subjects and animals. Inhibition of ACE results in decreased plasma angiotensin II which leads to decreased vasopressor activity and a decreased aldosterone secretion. Although ACE inhibitors are clinically effective, their use has been hampered by adverse effects such as temporary and reversible decline in glomerular filtration rate (GFR), angioedema and cough; which are thought to be caused due to inhibition of aldosterone synthesis and release (Pitt et al., 1997 and Garvas, 1999). Moreover, with prolonged use, the effectiveness of ACE inhibitors may be attenuated by generation of angiotensin II through non-ACE pathways or by up regulation of angiotensin I because of stimulation of rennin secretion. Several clinical trials (Anderson et al., 2000; Stigant et al., 2000; Brenner et al., 2001; Lewis et al., 2001) have however, established that these limitations of ACE inhibitors may be circumvented by use of the newer ARA.

To find evidence regarding the involvement of ACE inhibitors and ARAs in prevention of hypertension and thereby metabolic syndrome, all the parameters of metabolic syndrome were analyzed once again; though in context with antihypertensive monotherapy this time.

2B.1: Anthropometric observations

Table.2B.1 demonstrates the anthropometric characteristics of all the study subjects chosen for this section. A total of 99 subjects were selected, out of which 79 were the newly diagnosed hypertensive subjects, and the remaining 20 were normotensive controls (C). The hypertensive population was further sub-divided into three categories: those receiving no medication (HT+ND) at all, those receiving ramipril (HT+R) (5 mg orally administered, once in a day) as a monotherapy, and finally those receiving losartan (HT+L) (50 mg orally administered, once in a day) as a monotherapy prescribed by their physicians, for past 5 months. The groups receiving losartan (HT+L) and ramipril (HT+R) as a monotherapy comprised of elder subjects, compared to those belonging to the no drug (HT+ND) as well as control group (C). No significant difference was observed in waist circumference of the three hypertensive groups. Thus it can safely be said that neither ramipril, nor losartan play any remarkable role in the improvement of waist circumference or bringing it to near normal levels as displayed by control group (C). Observed reduction in systolic and diastolic blood pressure as a result of ramipril and losartan monotherapy, are also indicative of the role of these anti-hypertensive drugs in ameliorating metabolic syndrome.

2B.2: HDL levels

Besides the lowering of blood pressure, certain anti-hypertensives may have different pleiotropic effects on the pathophysiology of metabolic syndrome (Brenner et al., 2001). Accordingly, serum HDL concentration was measured for all the subjects involved. Fig.2B.2 put across no major variation in HDL levels of any of the group and thus suggests no significant role of ARA and ACE inhibitors in altering the lipid profile of hypertensive subjects. This result is in concordance with the previous reports suggesting only marginal effects of these drugs on lipid profile (Sica and Bakris, 2002).

2B.3: TGs levels

Akin to the alteration in HDL, increment of TGs levels is also an important indication of metabolic syndrome. As conceived in the former result, that showed no remarkable change in HDL even after ramipril or losartan monotherapy, the present result (fig.2B.3) also depicts that none of the anti-hypertensives have any beneficial effect over dyslipidemia. It is clearly evident that the TGs levels of non-treated (HT+ND), ramipril treated (HT+R), as well as the losartan treated group (HT+L) was almost the same but higher than that observed for the control group (C). Thus, it greatly emphasizes the need for some combination therapy, where drugs specifically capable of reducing lipid disorder should be used in addition to these ARA or ACE inhibitors in order to obtain a preventive measure for metabolic syndrome.

2B.4: Lipid peroxidation

Hypertension is a risk factor for atherogenesis (Kannel and Sorlie, 1975). The increased risk of CVD in hypertensive subjects correlates with blood pressure and may be linked to other factors including oxidative modification and accumulation of lipids in the vascular wall (Allemann and Weidmann, 1995). The effect of both ramipril and losartan was thereby observed against the concentration of lipid peroxides in the oxidized sdLDL of each subject involved in this study. Fig.2B.4 demonstrates the lowest lipid peroxidation in normotensive control subjects (C) and highest in the hypertensive subjects with no medication (HT+ND). However, it is clearly depicted that both ramipril (HT+R) and losartan (HT+L) monotherapy succeeded in reducing the amount of lipid peroxidation in hypertensive category. Thus, suggesting an important contribution of these anti-hypertensives towards suppression of oxidative stress, a major culprit of metabolic syndrome.

Parameters	C (n=20)	HT + ND (n=19)	HT + R (n=28)	HT + L (n=32)
Males (n) Females (n)	9 11	10 9	13 15	19 13
Age (Yrs)	33 ± 2.1	39 ± 3.6	42 ± 2.8	44 ± 3.2
WC (cm)	86 ± 1.3	98 ± 2.3	96 ± 3.1	95 ± 3.2
BP (mm Hg) (Sys) (Dia)	126 ± 1.3 75 ± 1.2	131 ± 2.3 78.8 ±1.5	115 ± 2.1 68.3 ± 2.0	117 ± 2.6 71 ± 1.6

Table.2B.1: Anthropometric observations in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The waist circumference (WC) and blood pressure (BP) for each subject was measured by the usual procedures as described in 'methods'. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

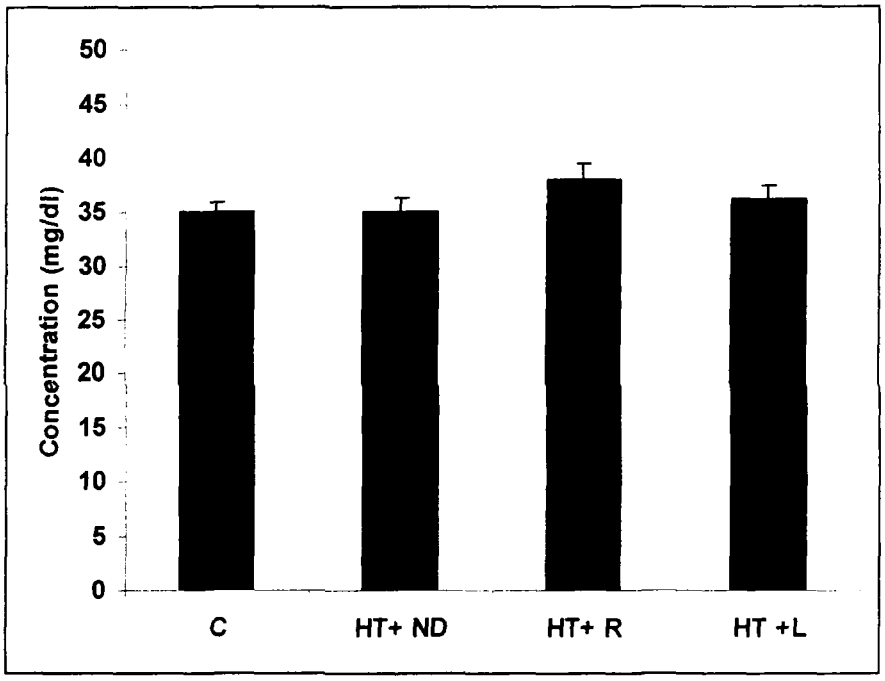


Fig.2B.2: HDL levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

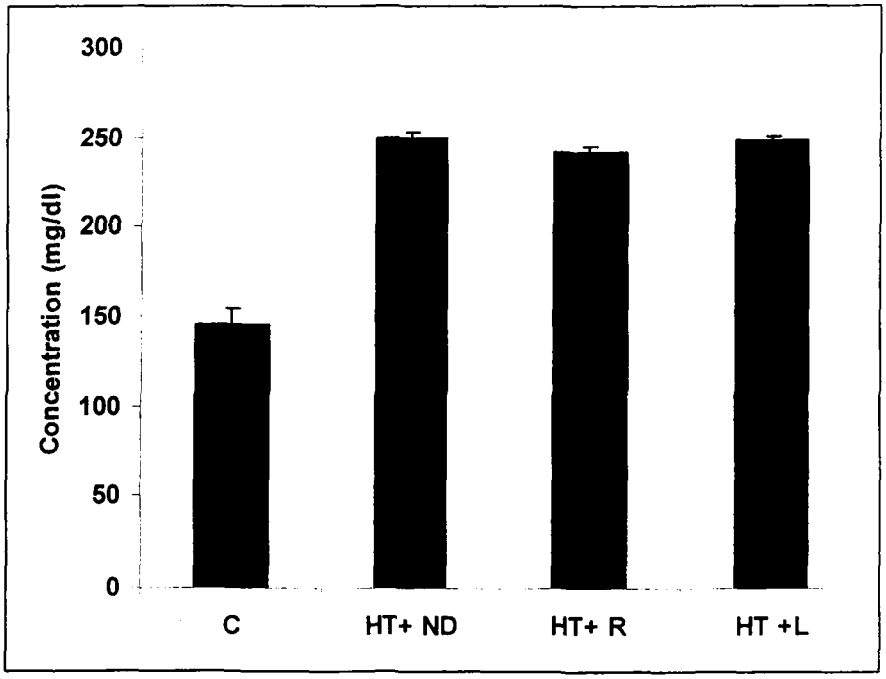


Fig.2B.3: TGs levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

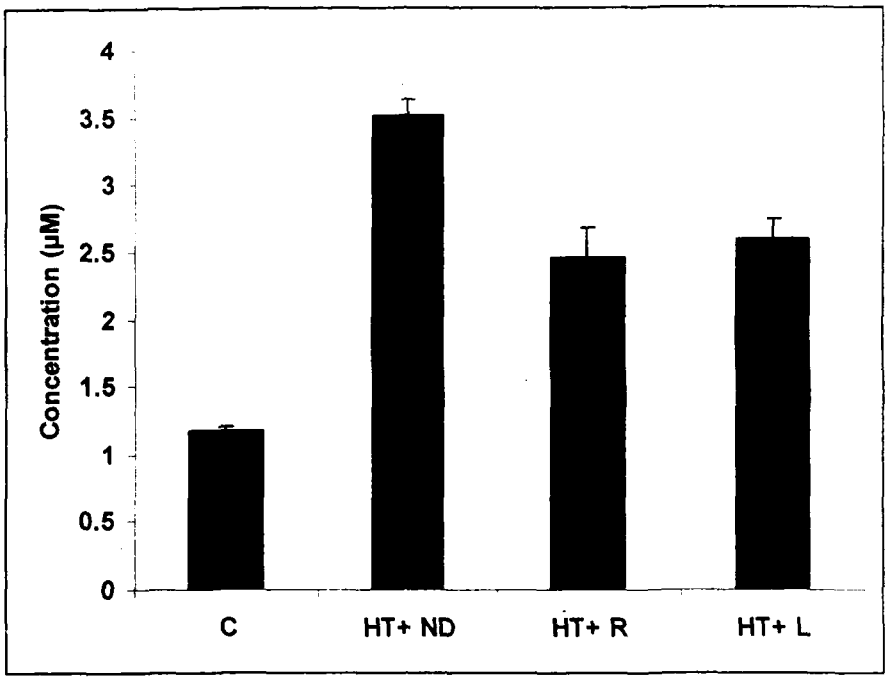


Fig.2B.4: Lipid peroxidation in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

2B.5: PON-1 activity

The activity as well as concentration of PON-1 has been reported to be decreased in CVD (Navab et al., 1997) and diabetes (Sakai et al., 1998). The results published from this laboratory have also proposed a diminished PON-1 activity in hypertension (Nishtha et al., 2008a). Following the same guideline, PON-1 activity of hypertensive subjects dependent upon ramipril or losartan was also measured. Surprisingly, the enzyme activity was found to be low in hypertensive subjects receiving no treatment (HT+ND) as compared to those receiving ramipril (HT+R) or losartan (HT+L) monotherapy (Fig.2B.5). Control group (C), however displayed the highest enzyme activity. This result is indicative of a plausible role of ARA and ACE inhibitors in maintaining the antioxidant status by upregulating the levels of PON-1. Thus, suggesting reduction in oxidative stress and the risk of metabolic syndrome.

2B.6: CRP and TNF-α levels

Rapidly evolving work now demonstrates that CRP has several direct effects at the level of the vessel wall (Pasceri et al., 2001). These observations along with basic research into the inflammatory mechanism of both diabetes and vascular dysfunction provide strong evidence that insulin resistance and atherosclerosis share a common inflammatory basis. Based on these evidences, CRP levels were evaluated in the normotensive as well as hypertensive subjects receiving either ramipril or losartan. This was performed in an attempt to verify the role of these drugs against inflammation, critically developing into metabolic syndrome. Fig.2B.6(a) represents a rise in CRP levels of hypertensive subjects receiving no treatment (HT+ND) while a significant drop in hypertensive subjects when treated with losartan (HT+L) and ramipril (HT+R). Nonetheless, the normotensive control group (C) revealed the lowest concentration of CRP.

Based on the evidences that elevated levels of another pro-inflammatory cytokine, TNF-α, also contribute substantially to metabolic syndrome (Devaraj et al., 2004), this research was further extended for the estimation of this component too. Fig.2B.6(b) witnesses a significant reduction in TNF-α levels of the losartan treated group (HT+L) as compared to that of no drug (HT+ND) and ramipril treated group (HT+R). The later ones, however showed almost similar values. The control group (C) showed an obvious lowest concentration of TNF-α. These results throw light on the pleiotropic effects of these drugs on the pathophysiology of metabolic syndrome.

2B.7: Apo-B levels and oxidation of sdLDL

Clinical assessment of the apo-B containing lipoproteins has fostered the investigations for measurement of standard lipids and their use as indices of atherogenic lipoprotein abnormalities of the metabolic syndrome (Austin et al., 1990). Consequently, apo-B levels were evaluated in all the subjects involved in this study and illustrated in fig.2B.7(a). The group containing normotensive control subjects (C) showed lowest concentration of apo-B; while no significant variation was depicted in the apo-B content of hypertensive subjects receiving no drug (HT+ND) and those receiving ramipril or losartan (HT+R, HT+L) as a monotherapy.

Similarly, fig.2B.7(b) represents the time taken for oxidation of sdLDL isolated from all the four categories. It is clearly indicated that the sdLDL isolated from all hypertensive subjects got oxidized in lesser duration of time as compared to that of normotensive control subjects (C). An almost equal amount of time was however noticed when rest of the three hypertensive groups (HT+ND, HT+R, HT+L) were compared to each other. Thus suggesting that ramipril and losartan are unable to reduce apo-B levels or more specifically, to create any qualitative changes in the lipoprotein profile of hypertensive subjects.

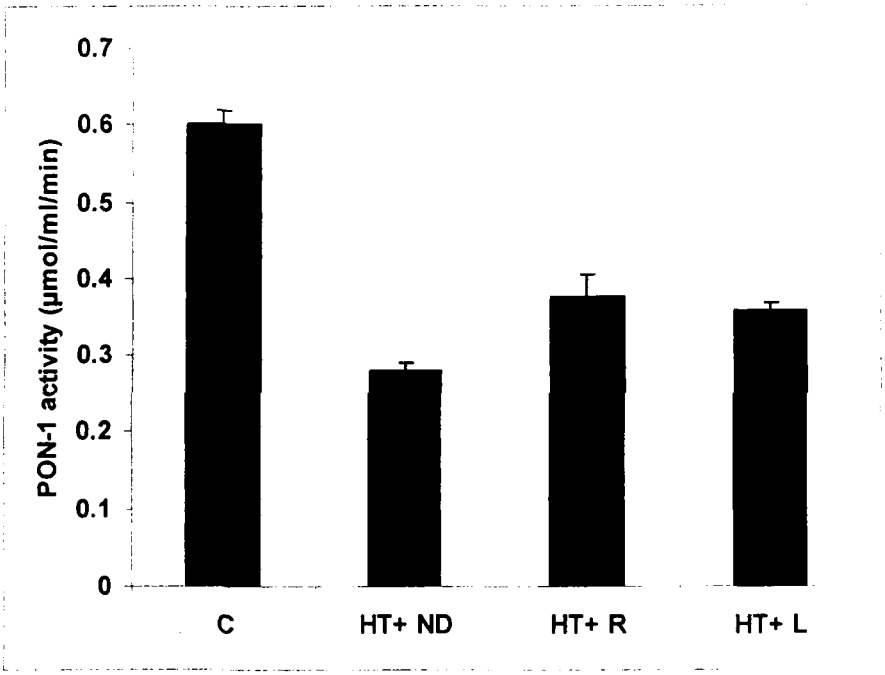


Fig.2B.5: PON-1 activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM $CaCl_2$ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

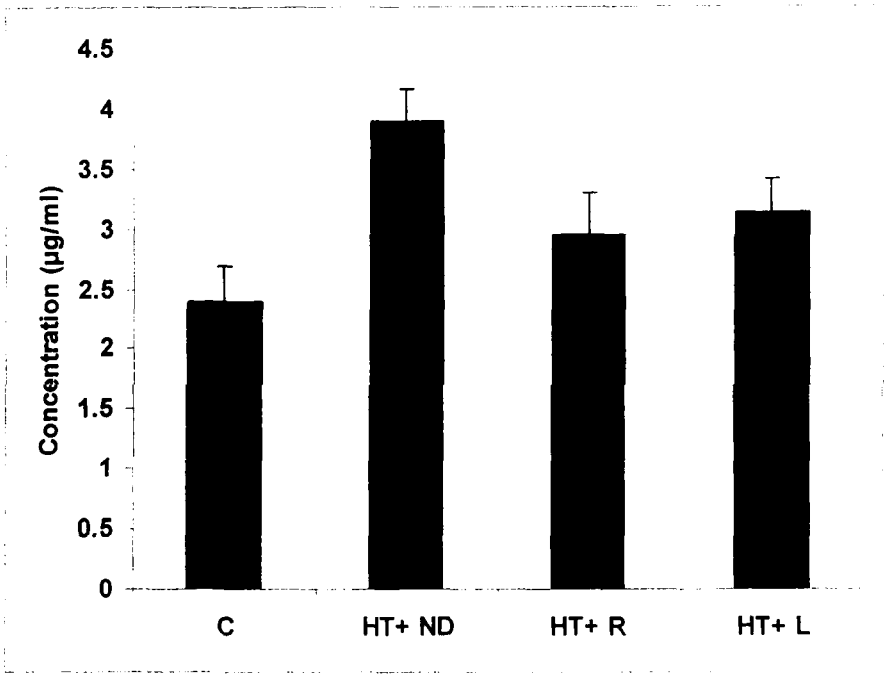


Fig. 2B.6(a): CRP levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

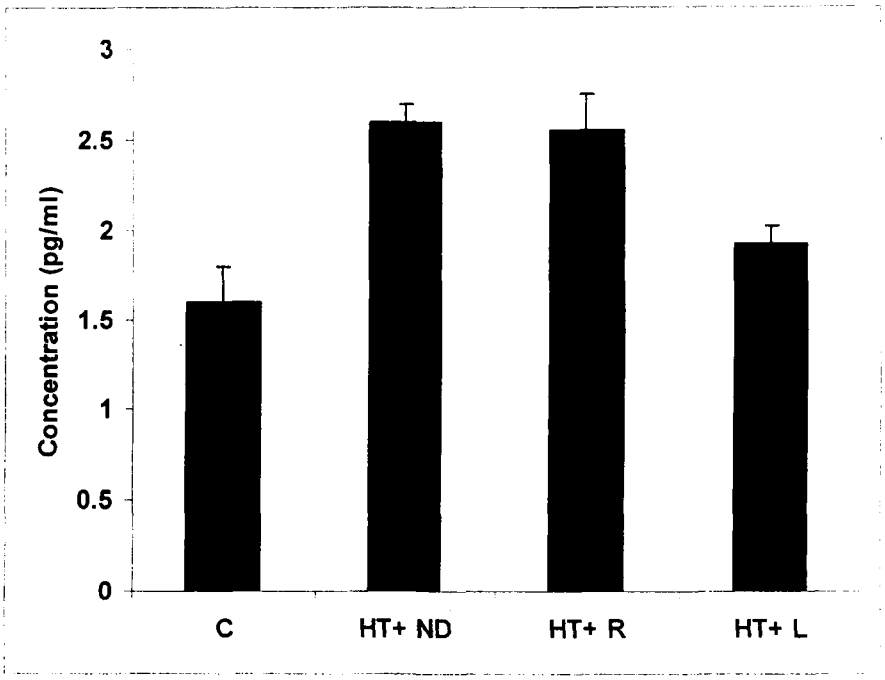


Fig.2B.6(b): TNF-α levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

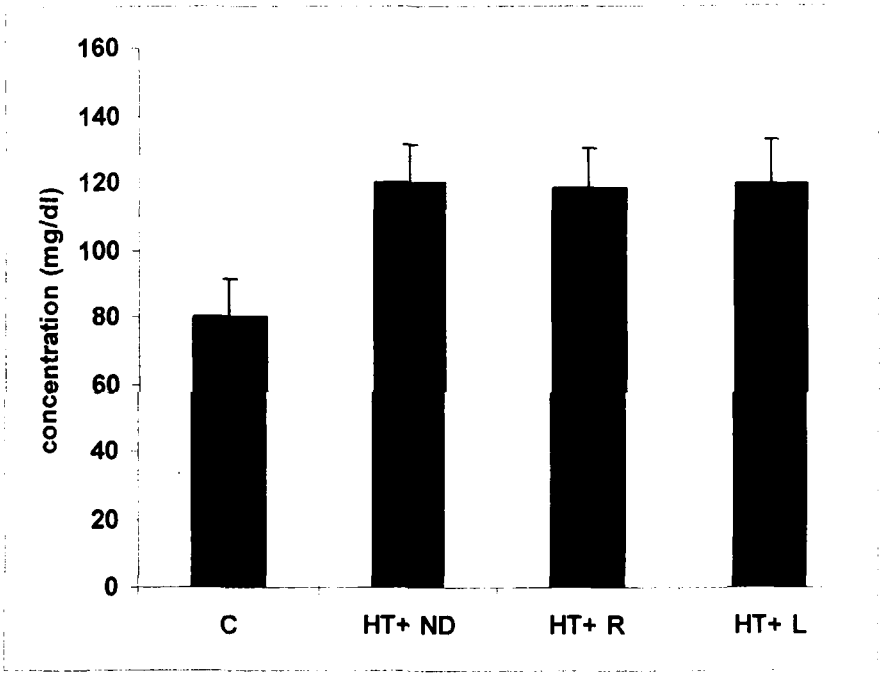


Fig.2B.7(a): Apo-B levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

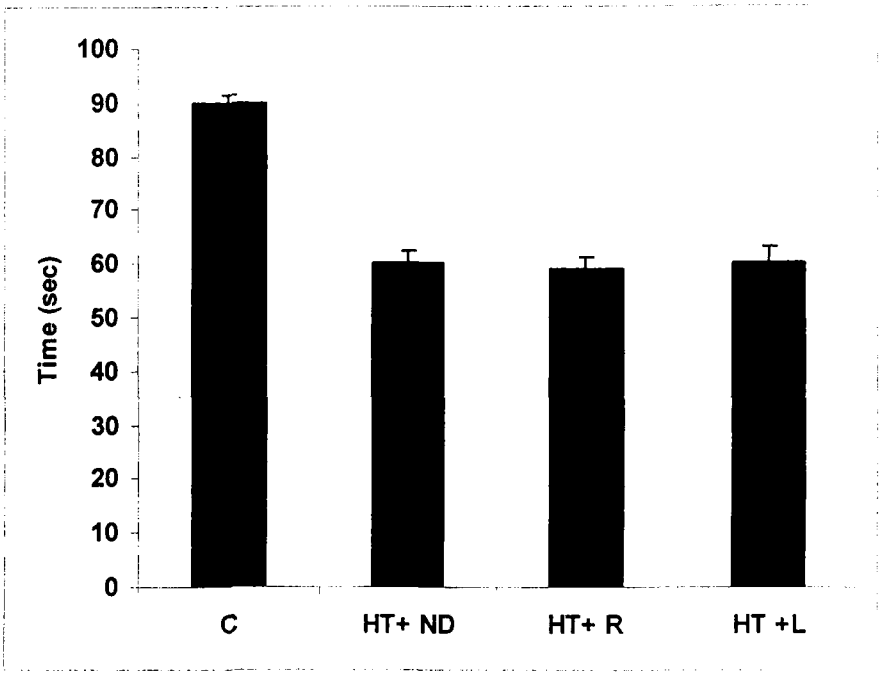


Fig.2B.7(b): sdLDL oxidation time of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM $CuCl_2$ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

2B.8: Oxidative stress marker MDA

Increased vascular oxidative stress could be involved in the pathogenesis of hypertension (Miyajima et al., 2007), a major risk factor for cardiovascular disease mortality. Also it has been reported that in essential hypertension ROS may increase due to a diminution of the activity of antioxidant enzymes (Pedro-Botet et al., 2000). To explore any protective role offered by ACE inhibitors or ARAs against oxidative stress, this study evaluated the levels of MDA, the oxidative stress damage index in hypertensive subjects. Fig.2B.8 demonstrates highly elevated MDA levels in the group of hypertensive subjects receiving no drug at all (HT+ND) as compared to the normotensive subjects (C). However, the treatment with ramipril alone (HT+R), significantly reduced the MDA levels. A relatively lesser amount of decrease was also noticed in the losartan treated group (HT+L). These findings are consistent to the previous reports suggesting reduction in oxidative stress and improvement in the endothelial function in hypertensive patients following ramipril and losartan monotherapy (Beatriz et al., 2007; Barath et al., 2006).

2B.9: SOD and Catalase activity

The occurrence of oxidative stress may arise from a primary decrease in the antioxidant defense system or from an elevation of ROS concentration. This derangement leads to oxidative damage to the structure of biomolecules, likely involving the antioxidant enzymes, thus contributing to the oxidative stress found in hypertensive subjects, but not in normotensive subjects (Rodrigo et al., 2007). As a consequence of decreased activity of antioxidant enzymes SOD and catalase, a reduction in the endothelium dependent vasodialation of the vascular smooth muscle cells of hypertensive subjects could be expected (Lassègue and Griendling, 2004). The treatment with anti-hypertensives such as ramipril or losartan may however provide a protection from these damaging consequences. Therefore the assays were performed for

determination of SOD as well as catalase activity in the hypertensive subjects receiving no drug and those receiving ramipril or losartan as a monotherapy. Fig.2B.9(a,b) reveal reduced activity of both SOD and catalase in the hypertensive group receiving no treatment (HT+ND) suggesting high oxidative stress. The treatment with ramipril (HT+R) and losartan (HT+L) however successfully replenished the antioxidant enzyme activities in the hypertensive subjects. Interestingly, the effect of ramipril therapy was more pronounced than that of losartan therapy, suggesting its greater antioxidant capacity. Similar protective effects of ramipril and losartan against hypertension have been reported earlier (Khattab et al., 2004; Barath et al., 2006) and this study also put forward an important role of ramipril in ameliorating oxidative stress.

2B.10: DNA damage

Earlier studies have shown an association between hypertension and the oxidation of LDL, and particularly the fact that its susceptibility to oxidation is greater in patients with essential hypertension than in normotensive subjects (Pierdomenico et al., 1998). It is suggested that oxidative modification of LDL could promote and accelerate the development of atherosclerosis (Witztum, 1994). Some studies have demonstrated the presence of DNA damage and DNA adducts in the atherosclerotic lesions and tissues (Binkova et al., 2002 and Martinet et al., 2002). The result presented in fig.2B.10 is consistent to the above reports, as a significant DNA damage was observed in hypertensive patients of all the three categories (HT+ND, HT+R, HT+L) as compared to the control group (C). However, the three groups of hypertensive subjects showed an almost equal extent of DNA damage; suggesting no beneficial role of ramipril or losartan against DNA damage, a major factor of metabolic syndrome.

2B.11: Correlation between NCEP/ATP III parameters and observed parameters

Present work focuses the effect of anti-hypertensive drugs on metabolic syndrome. The NCEP/ATP III components (HDL, TGs, and BP) as well as the parameters observed (CRP, TNF-α and apo-B levels together with sdLDL oxidation time, lipid peroxidation, PON-1 activity, and extent of DNA damage), have been employed to ascertain the potential of metabolic syndrome in hypertensive and normotensive subjects chosen for this study. However, to find a relationship between these factors and NCEP/ATP III parameters in hypertensive subjects with the administration of ramipril or losartan therapy, Pearson's correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.2B.11 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs together with diastolic BP, (ii) lipid peroxidation and HDL, (iii) CRP and HDL, (iv) sdLDL oxidation time and HDL (v) TNF-α and TGs, (vi) apo-B and HDL (vii) DNA damage and HDL. Thus it may be suggested that reduction in BP and TGs levels upon ramipril or losartan monotherapy instigate the elevation of PON-1 activity in hypertensive subjects. Also the increased HDL concentration may facilitate the decrease in lipid peroxidation, levels of CRP and apo-B along with the amendment of DNA damage as well as the time taken by sdLDL for oxidation. Changes in the TNF-α level however, was shown to be related with the reduction of TGs levels. This implies that improvement in BP, TGs as well as HDL levels on account of ramipril or losartan monotherapy play a pivotal role in regulation of several other metabolic syndrome parameters in hypertensive subjects.

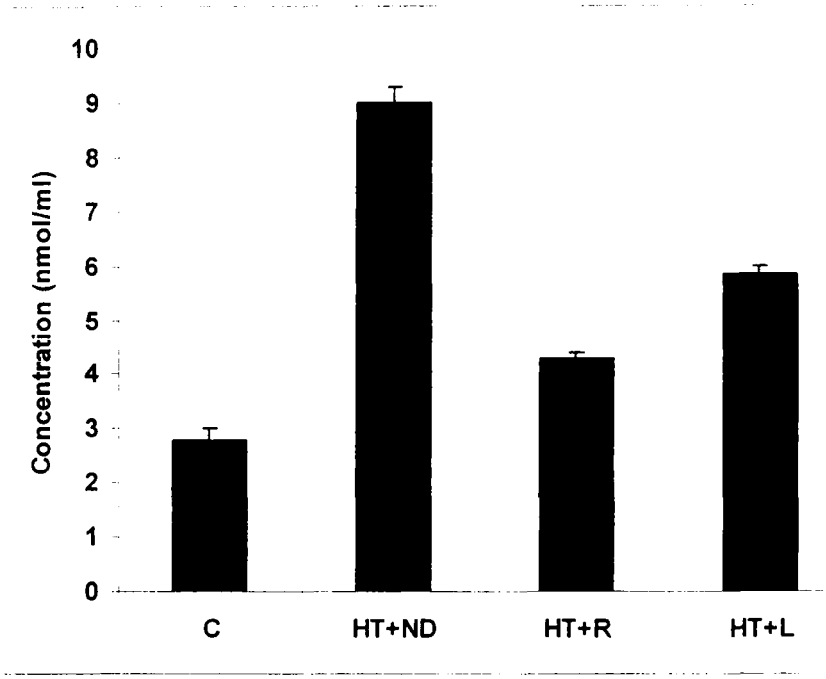


Fig.2B.8: MDA levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in 'methods'. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

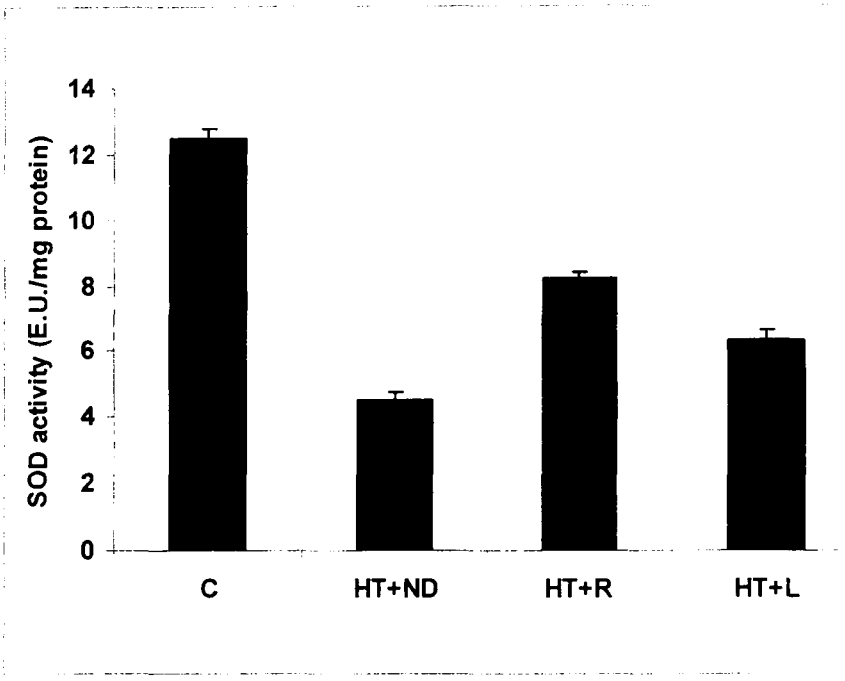


Fig.2B.9(a): SOD activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The assay medium in a final volume of 3 ml consisted of 50 µl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

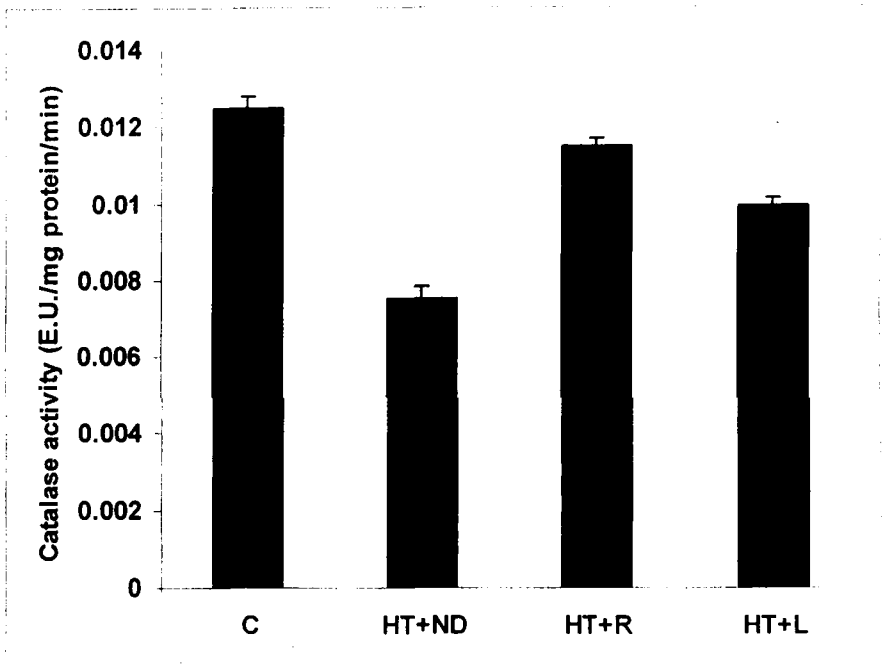


Fig.2B.9(b): Catalase activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H_2O_2). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml H_2O_2 (30 mM) and 50 µl sample. One enzyme unit is defined as the amount of enzyme decomposing 1 µM H_2O_2 per min at 25°C. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

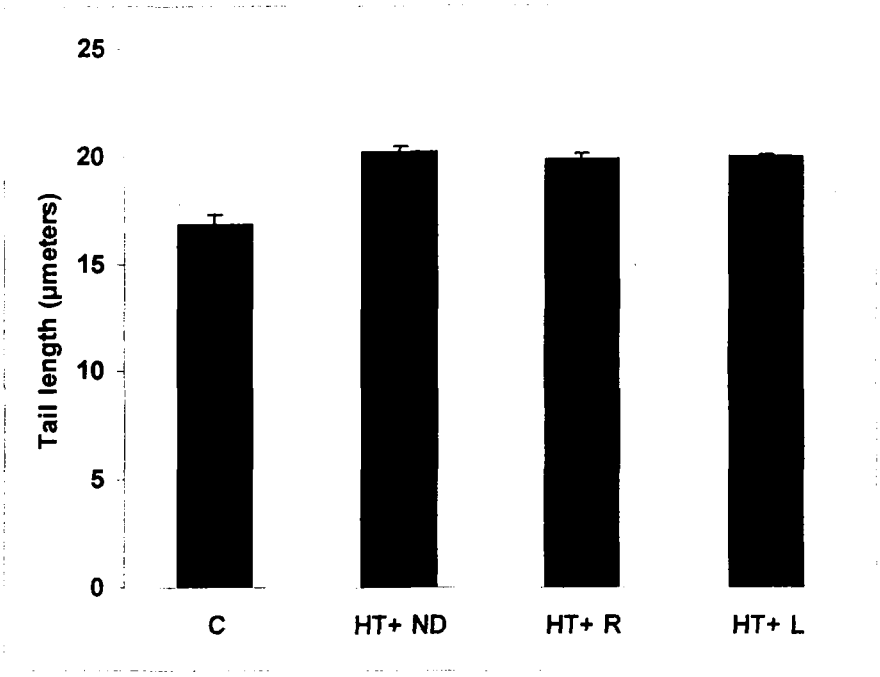


Fig.2B.10: DNA damage in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

DNA breakage in the lymphocytes isolated from each subject was measured by comet assay proposed by Singh et al. (1988) as described in 'methods'. Comet tail length (µmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

C	Control
HT + ND	Hypertensive + No Drug
HT + R	Hypertensive + Ramipril
HT + L	Hypertensive + Losartan

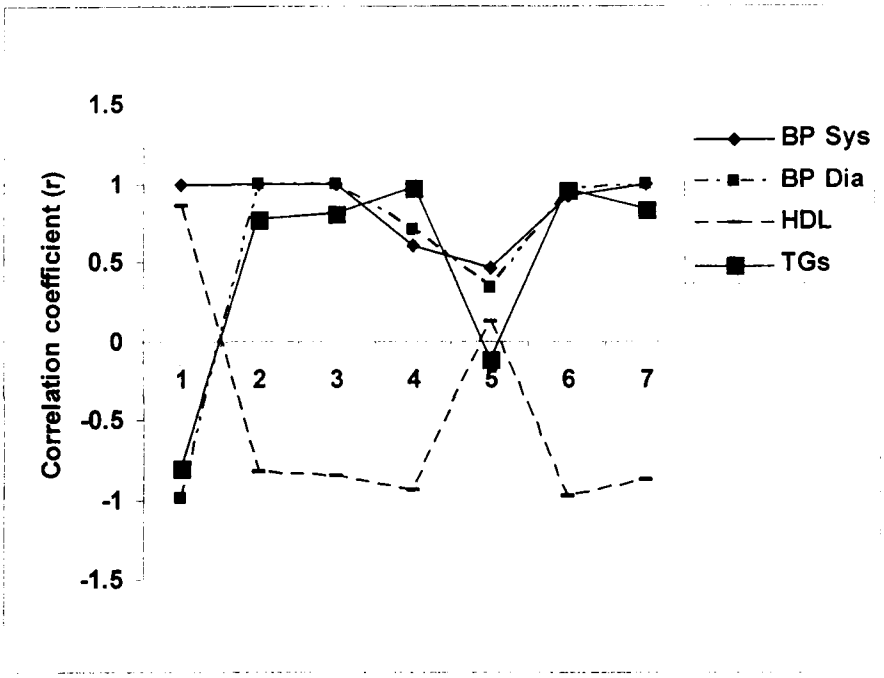


Fig.2B.11: Correlation between NCEP/ATP III parameters and observed parameters with ramipril or losartan monotherapy.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed in hypertensive subjects with the administration of ramipril or losartan therapy. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1 PON-1 activity
2 Lipid peroxidation
3 CRP concentration
4 sdLDL oxidation time
5 TNF-a concentration
6 Apo-B concentration
7 DNA damage

Chapter 3

Treatment of Metabolic Syndrome (Animal Studies)

Hitherto, it has been recognized that diabetic subjects exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of antioxidative defense system and promoting the generation of free radicals. To this end, it has become clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications that may also lead to metabolic syndrome. There are evidences suggesting that antioxidants, such as α-tocopherol (vitamin E), have potential benefits with respect to CVD. Alpha-tocopherol has been shown to decrease lipid peroxidation, inhibit platelet adhesion, aggregation and smooth muscle cell proliferation, to exert anti-inflammatory effect on monocytes and to improve endothelial function (Harris et al., 2002). Animal studies have shown that vitamin E protects development of cholesterol-induced atherosclerosis by inhibiting protein kinase C activity in smooth muscle cells *in vivo* (Kartal et al., 2003).

Ascorbic acid (vitamin C) is the main water-soluble antioxidant in human plasma (Frei et al., 1990). Earlier it was reported that long-range, heavy dose ascorbic acid therapy tends to normalize considerably, but not completely, the disturbances in certain blood fat metabolism factors, such as total cholesterol, triglycerides and lipoprotein lipase (LPL) activity (Sokoloff et al., 1967). Several studies have demonstrated that ascorbic acid improves endothelial-dependent vasodilatation in hypertension and diabetes. It has been proposed that such effects may be due to scavenging of superoxide anions and its reactive oxygen intermediates (Ting et al., 1996).

Earlier the role of metformin and insulin was verified against diabetes and metabolic syndrome, no significant improvement was observed though. This study therefore, is an attempt to investigate whether the supplementation of vitamin C or E in diabetic animals could be an alternative to prevent oxidative stress and metabolic syndrome or not. For this purpose, a total of 12 rabbits were distributed into four experimental groups of three rabbits per group: non-diabetic (control, G1); alloxan-induced

diabetic (G2); diabetic supplemented with 150 mg/kg body weight of vitamin C (G3) and diabetic supplemented with 1000 I.U./kg chow of vitamin E (G4), administered daily as an oral supplementation via gavage for a period of two weeks. Thereafter, 5 ml fasting blood was collected from each animal and serum was isolated to determine all the parameters of oxidative stress and metabolic syndrome.

3.1: Elevated parameters of metabolic syndrome

3.1(a): FBG levels

It has been recognized previously that an increase in plasma glucose to above normal levels typically develops late in the course of metabolic syndrome (Genuth et al., 2003). This study therefore, employs the measurement of FBG in all the four animal groups involved. Fig.3.1(a) demonstrates, that the FBG level was highest for the diabetic group (G2) and lowest for the non-diabetic, control group (G1). The groups supplemented with antioxidants, vitamin C and vitamin E (G3 and G4), however attained an intermediate level, with the lowest FBG level in G3, among treated groups. This is consistent with the previous findings suggesting that antioxidants have potential benefits with respect to CVD (Vieira and Vianna, 2005).

3.1(b): TGs levels

Altered metabolism of triglyceride-rich lipoproteins and their elevated levels in serum can contribute substantially to the risk of ischemic heart disease in patients with metabolic syndrome (Krauss and Siri, 2004). In light of these observations, serum TGs levels were also estimated in the present study. Fig.3.1(b) demonstrates that the concentration of TGs was highest in diabetic group (G2), signifying atherogenic dyslipidemia and a possibility for the development of metabolic syndrome. Further, the supplementations of vitamin C and Vitamin E (G3 and G4) to the diabetic rabbits lead to the reduction in their TGs levels. This finding is in agreement to the proposal

drawn by Vieira and Vianna (2005) that vitamin supplementation may be used as an alternate therapy to treat lipid disorder for diabetic subjects.

3.1(c): Apo-B levels

Following the elevation in TGs levels, a somewhat greater portion of apo-B is observed in VLDL. There is growing evidence that all apo-B containing lipoproteins are atherogenic and elevated apo-B levels cause atherogenic dyslipidemia (Grundy, 2004). Consequently, apo-B levels were determined for all groups involved in this study and illustrated in fig.3.1(c). It was observed that diabetic group (G2) contained highest apo-B levels followed by vitamin E (G4) and vitamin C (G3) supplemented groups. The lowest apo-B levels attained by G3 suggest that vitamin C may play an important role in regulating metabolic syndrome and associated abnormalities.

3.1(d): Lipid peroxidation

Diabetes is known to be associated with increased oxidative stress *in vivo* (Zozulinska et al., 1996). The increased oxidative stress together with poor metabolic control enhances lipid peroxidation in diabetic subjects, which has been proposed to be related with aetiology of diabetic complications or metabolic syndrome (Armstrong and Al-Awadi, 1991). In the present study, concentration of lipid peroxides was determined in the oxidized sdLDL isolated from the serum of each animal, by the method proposed by el-Saadani et al. (1989). Results obtained in fig.3.1(d) conceive that the animals belonging to diabetic group (G2) had the greatest amount of lipid peroxides; whereas those supplemented with antioxidants such as vitamin C or vitamin E (G3, G4) had lesser amounts of lipid peroxides in their serum. This result rationally explains that lipid peroxidation may occur due to depletion of endogenous antioxidants in sdLDL. Hence, the inverse relationship between plasma levels of dietary antioxidants and the risk of metabolic syndrome observed in several epidemiological studies can be clearly understood.

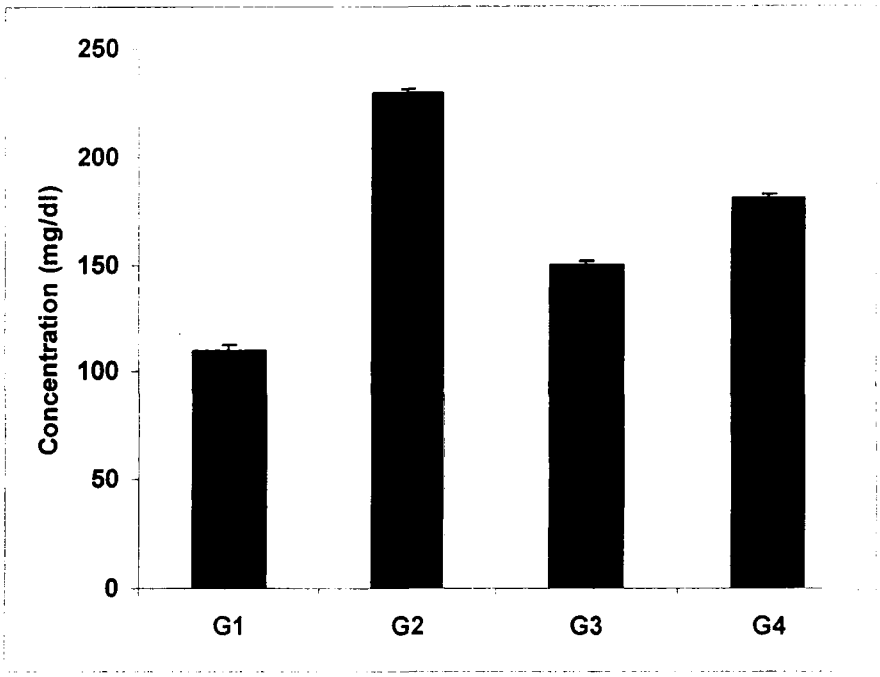


Fig.3.1(a): FBG levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The FBG level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 200 mg/dl. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

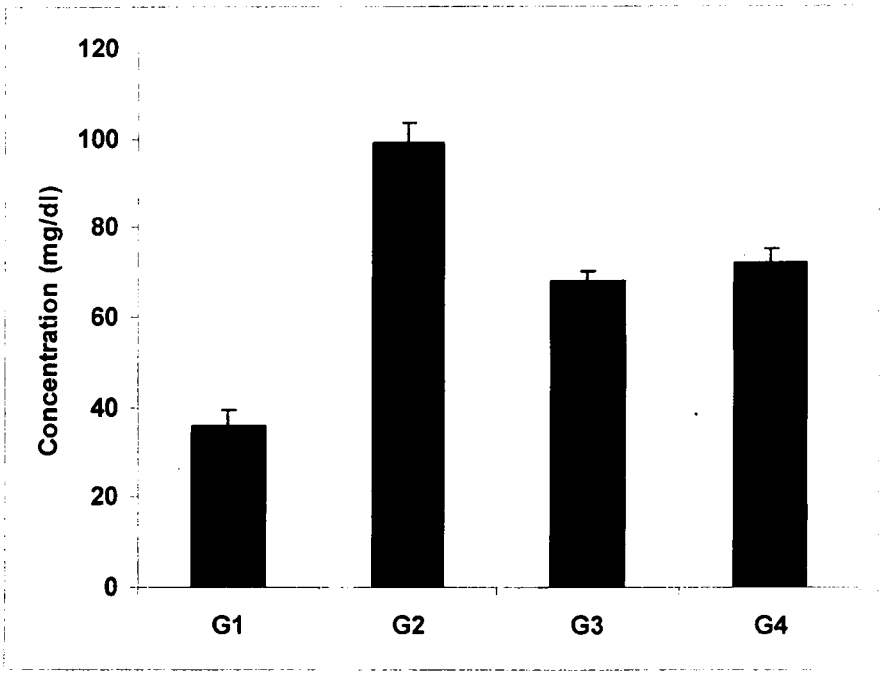


Fig.3.1(b): TGs levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

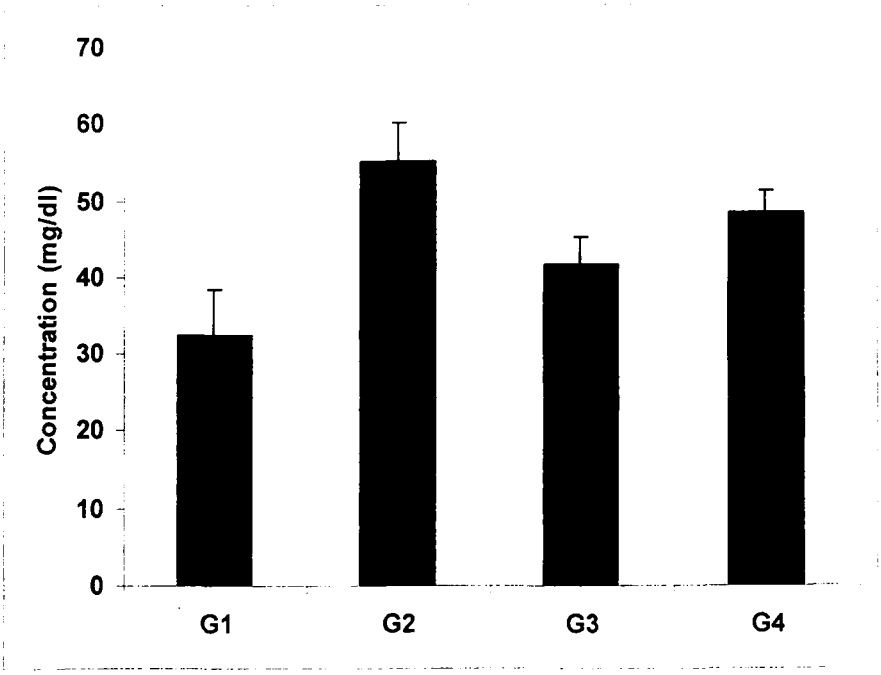


Fig.3.1(c): Apo-B levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

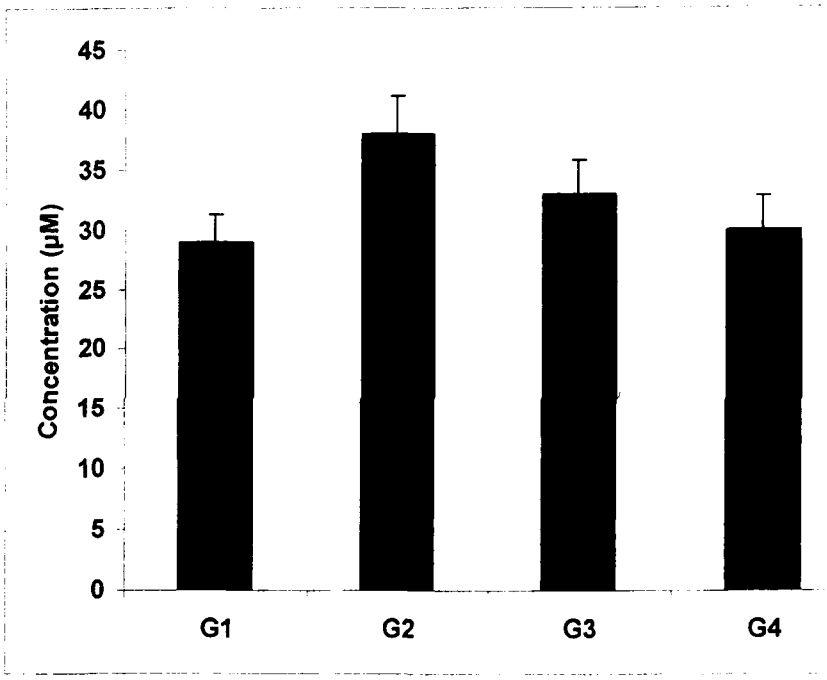


Fig.3.1(d): Lipid peroxidation in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

3.1(e): CRP levels

Persons with metabolic syndrome commonly have high levels of CRP (Ridker et al., 2003). Evidence is growing that an elevation of serum CRP levels predicts the development of type 2 diabetes and occurrence of major CVD events (Pradhan et al., 2001). In order to validate the necessity of estimating this inflammatory marker as a component of metabolic syndrome, the serum CRP levels were measured. Fig.3.1(e) shows that maximum concentration of CRP was found in the diabetic group (G2), followed by vitamin E and C supplemented groups (G4, G3) and finally the non-diabetic, control group (G1). The surprisingly reduced CRP concentration in the antioxidant supplemented groups indicates the role of these vitamins in warding off the complications associated with metabolic syndrome. Numerous studies have confirmed that CRP levels are elevated in patients with metabolic syndrome (Tracy, 1999; Rifai and Ridker, 2001; Jialal and Devaraj, 2003) but none, best to our knowledge, have yet reported the suppression of CRP levels after antioxidant supplementation. Nonetheless, Frohlich et al. (2000) have demonstrated the association between CRP and the features of metabolic syndrome in a population-based study. It was concluded that mean CRP levels increased steadily as the number of metabolic abnormalities increased.

3.1(f): TNF-α levels

TNF-α is another proinflammatory cytokine secreted by monocytes-macrophages, endothelial cells, and to a large extent, by adipocytes. Several studies have shown that levels of TNF-α are an important regulator of insulin sensitivity (Hotamisligil, 1993) and that neutralization of TNF-α improves insulin sensitivity in fa/fa rats but not in obese humans with diabetes (Arner, 2003 and Kern et al., 1995). The results avowed in fig.3.1(f) coherently states that TNF-α levels were raised in the group of diabetic rabbits (G2). The levels were reduced significantly, in the groups supplemented with vitamin C (G3) and vitamin E (G4). It may be thus assumed that treatment with vitamins or

antioxidants suppress the exacerbation in insulin resistance and other metabolic abnormalities that are probably caused by elevation of proinflammatory cytokines and other factors of metabolic syndrome.

3.2: Declined parameters of metabolic syndrome

3.2(a): HDL levels

An inverse relationship between the levels of HDL and the risk of developing premature CVD has been a consistent finding in many prospective population studies. In several of these studies, the level of HDL has been the single most powerful lipid predictor of future CVD events (Assmann et al., 1998; Manninen et al., 1992; Stamler et al., 1986). The NCEP/ATP III criterion also puts forth the importance of decreased HDL concentration in diagnosing metabolic syndrome. Consequently, HDL concentration was determined in all the four groups participating in this study. Fig.3.2(a) shows that the diabetic group (G2) has lowest concentration of HDL followed by the vitamin E group (G4), vitamin C group (G3), and finally the control group (G1). Thus, above result indicates the efficacy of vitamins in improving HDL profile; thereby supporting the hypothesis of Gordon et al (1989) which suggests that, for every 1 mg/dl (0.025 mM) increase in HDL-C, the CVD risk is reduced by 2% to 5%.

3.2(b): PON-1 activity

Paraoxonase-1 (PON-1) is an HDL-associated enzyme that possesses antioxidative properties. In addition, diabetes is characterized by increased oxidative stress and decreased PON-1 activity. The mean serum PON-1 activity for G1, G2, G3 and G4 is demonstrated in fig.3.2(b). The highest and the lowest enzyme activity corresponding to the control group G1 and diabetic group G2, strongly suggest the reduction of arylesterase activity of PON-1 during oxidative stress. However, a noticeable elevation in the enzyme activity of the groups supplemented with vitamin C and E (G3, G4)

indicates the protection offered by antioxidants, possibly through maintaining the balance of excess free radicals and antioxidant defenses. This result also highlights the significance of decreased oxidative stress and increased PON-1 activity in recuperating diabetes as well as metabolic syndrome. Rozenberg et al. (2008) have reported that increasing PON-1 expression in mice could attenuate the development of diabetes, a phenomenon which could be attributed to the antioxidative properties of PON-1 and decreased oxidative stress.

3.2(c): sdLDL oxidation

Previous reports have established that not only quantitative but also qualitative changes to lipoprotein profiles contribute to the increased incidence of coronary diseases (Gardner et al., 1996). Another important contributor to increased coronary risk is oxidative stress (Chisolm and Steinberg, 2000). In addition, there may be a pathological link between these parameters as modified forms of low density lipoproteins such as sdLDL are more susceptible to oxidative stress (de Graff et al., 1991). Similarly, fig.3.2(c) demonstrates the average time required for the oxidation of sdLDL. It is clearly depicted that sdLDL of diabetic group (G2) got oxidized earliest as compared to other experimental groups. One means by which sdLDL could increase the risk of vascular diseases is their greater susceptibility to oxidation (de Graff et al., 1991). Diabetes is associated with increased oxidative stress (Ceriello, 2003b) and anti-oxidant protective mechanisms are suggested to be sub-optimal in diabetes and metabolic syndrome (Ford et al., 2003). Eventually, the time required for oxidation of sdLDL in vitamin supplemented groups (G3 and G4) was more than that required by the diabetic group receiving no such supplementation. The sdLDL isolated from vitamin C supplemented group however, procured maximum time to get oxidized, conferring a better preventive measure for diabetes as well as metabolic syndrome.

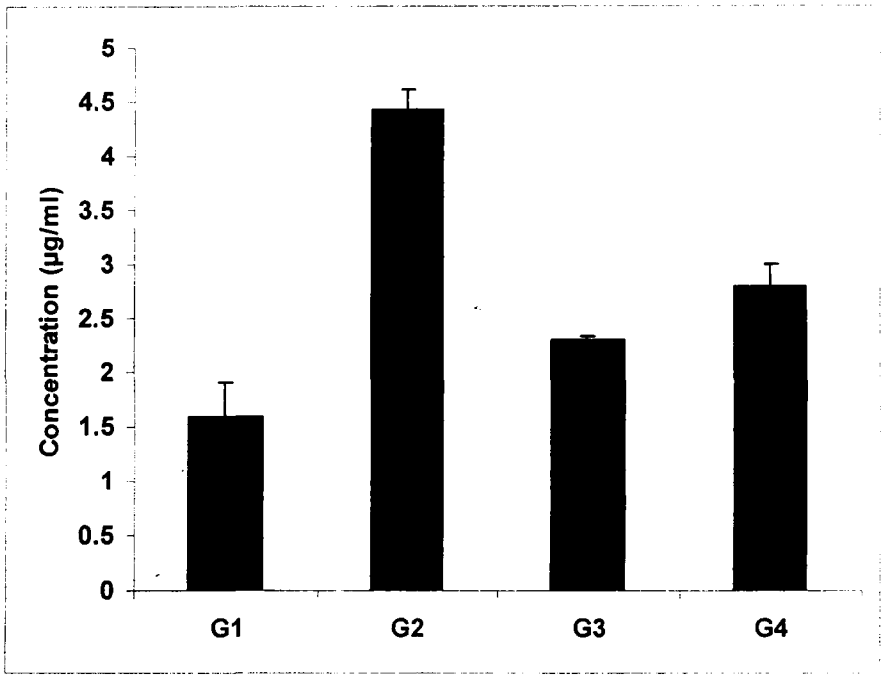


Fig.3.1(e): CRP levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

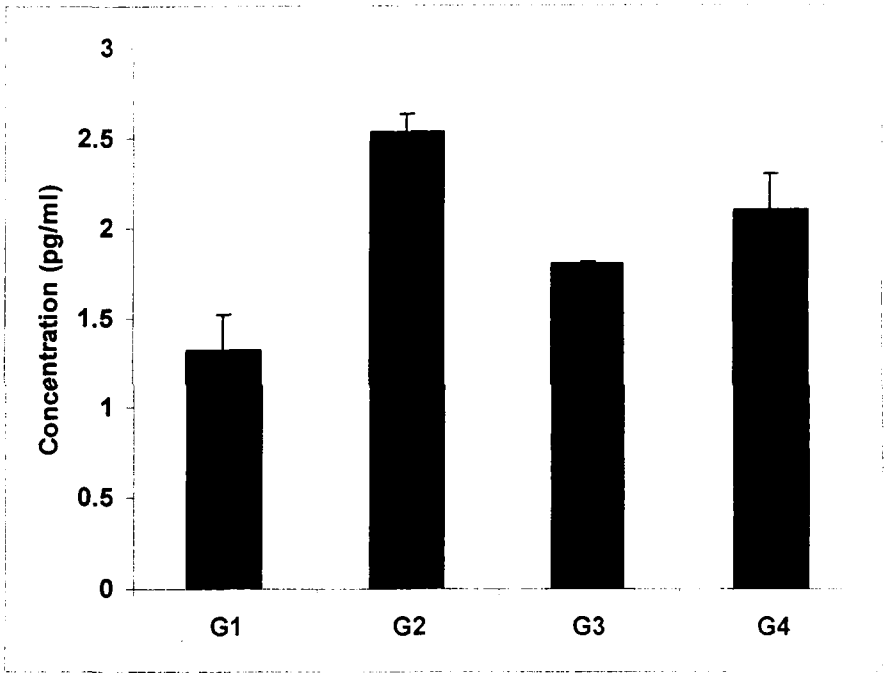


Fig.3.1(f): TNF-α levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

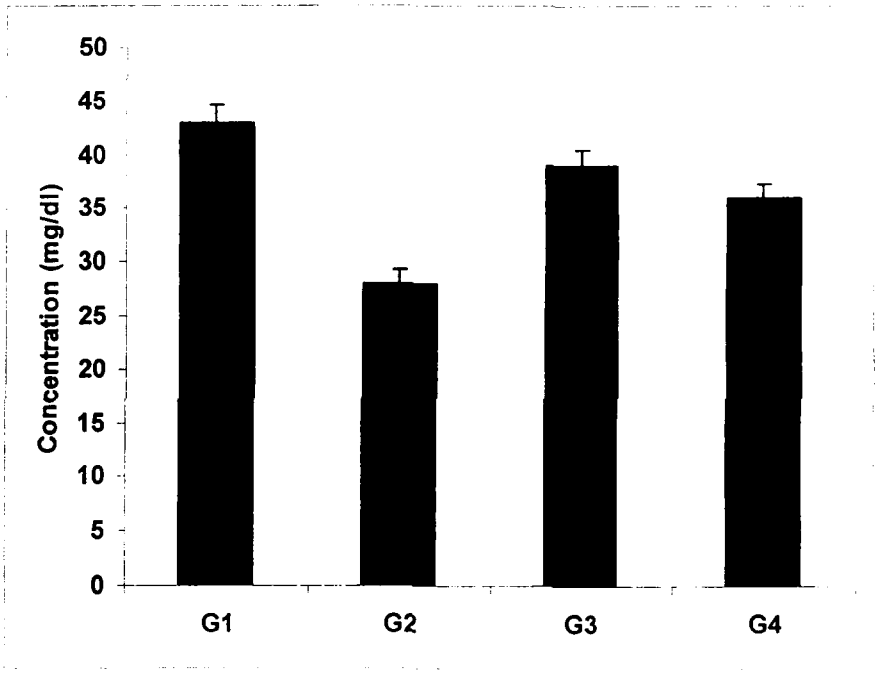


Fig.3.2(a): HDL levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

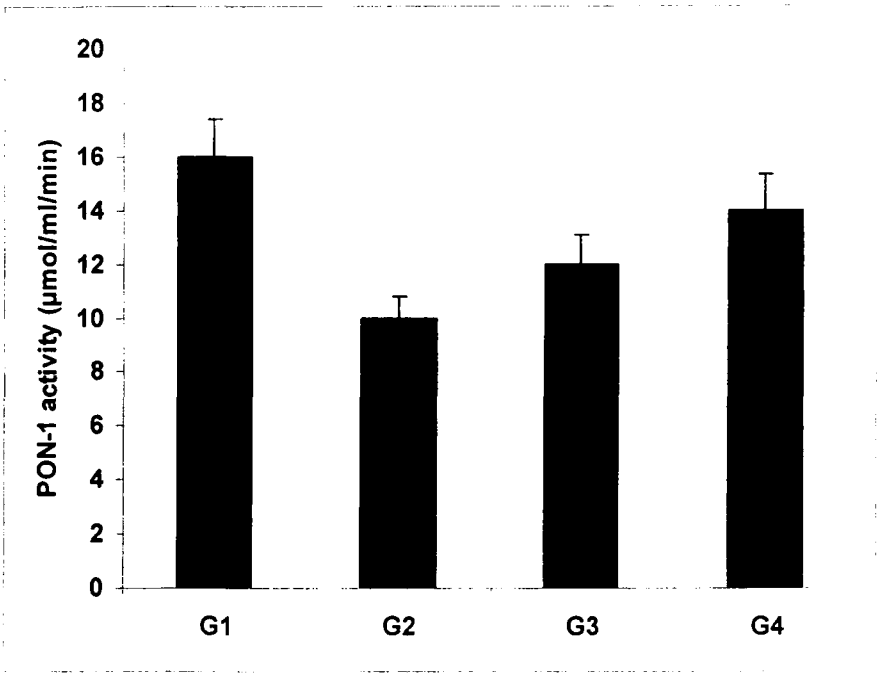


Fig.3.2(b): PON-1 activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM $CaCl_2$ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

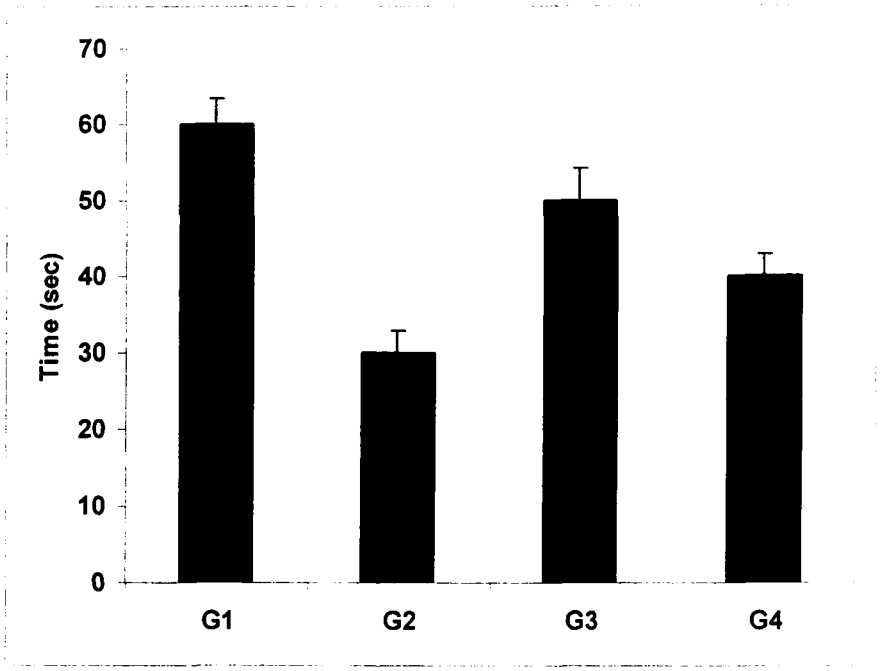


Fig.3.2(c): sdLDL oxidation time of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM $CuCl_2$ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

3.3: Oxidative stress marker MDA

In recent years it has been known that, the most important factor that increases the free radicals production in diabetes is the hyperglycemic status, which can induce damage such as lipid peroxidation. Present approaches to diabetic therapy involve mainly drugs enhancing insulin secretion or signaling as well as inhibitors of endogenous glucose production (Aguirre et al., 1998), while the role of antioxidants as agents important for restoring the redox balance of the organism is still underestimated. This study therefore focuses the role of vitamin C or E alone in suppressing oxidative stress and related anomalies in alloxan-induced diabetic rabbits. Fig.3.3 illustrates the highest MDA levels, in diabetic animals (G2). Besides, it was also shown that supplementation with vitamin C (G3) as well as vitamin E (G4) successfully reduced the MDA levels. Present results hence, signify the involvement of antioxidant therapy in ameliorating oxidative stress.

3.4: SOD and Catalase activity

Under normal circumstances, free radicals that are produced through biological processes and in response to exogenous stimuli are controlled by various enzymes and antioxidants in the body. SOD and catalase are paradigm of such enzymes and generate the antioxidant defense system (Cheryl et al., 1996). A group of researchers have previously reported that a single dose of alloxan produced a decrease in the liver and pancreatic SOD and catalase activities during the development of alloxan-induced diabetes (Hamden et al., 2008). Akin to these findings, our results represented in fig.3.4(a) and (b) also demonstrate changes in the activity of antioxidant enzymes SOD and catalase, when verified in the serum of diabetic rabbits after the supplementation of vitamin C or E. It is elucidated that the specific activity of both SOD and catalase drop off significantly with diabetes and raised concomitantly after vitamin therapy as seen in G2, G3 and G4.

Nonetheless, the extent of recovery was slightly greater in G3, the vitamin C supplemented group, as compared to G4, the vitamin E supplemented group; suggesting it to be more effectual against oxidative stress.

3.5: DNA damage

Increased blood glucose levels stimulate the production of ROS, which can cause damage to biological macromolecules such as proteins and DNA (Bonnefont-Rousselot, 2002). In the present study, DNA damage was observed in the lymphocytes of alloxan-induced diabetic rabbits with or without the supplementation of antioxidants. The damage (expressed as tail length, μmeters) was measured in all the four groups G1, G2, G3 and G4 via comet assay (as described in 'methods'). The representative photographs displaying the images of comets obtained after DNA damage before and after the supplementation of vitamin C or E is shown in fig.3.5(a). The corresponding tail length was also determined and represented in fig.3.5(b). Maximum DNA damage was observed in diabetic rabbits (G2) depicting the association of diabetes with increased oxidative stress. The antioxidants supplemented groups however experienced a lesser extent of DNA damage and it was observed that the reduction was more pronounced in vitamin C supplemented group (G3) as compared to the vitamin E supplemented group of diabetic rabbits. This result justifies the scavenging of free radicals by these vitamins and reduction of ROS, thereby ameliorating DNA damage in diabetes and metabolic syndrome.

3.6: Correlation between DNA damage and metabolic syndrome

A number of studies have reported the relation between DNA damage and diabetes (Hannon-Fletcher et al., 2000; Lima et al., 2007; Demirbag et al., 2005). To determine the association of DNA damage with metabolic

syndrome, Pearson's correlation analysis was performed. The correlation coefficient (r) obtained is an easy indicator of the role of elevated as well as declined parameters of metabolic syndrome in carrying out DNA damage. Fig.3.6 represents the result obtained after the correlation analysis and indicates positive correlation coefficient for the elevated factors and negative correlation coefficient for the declined factors of metabolic syndrome. This is an indication of enhanced DNA damage with increase in CRP, TNF-α, lipid peroxides, TGs and apo-B levels together with an increment in blood glucose. However, the negatively correlated factors propose an inverse relationship and explain the association of DNA damage with a decline in PON-1 activity, HDL concentration and the time required for oxidation of sdLDL. It can thus, safely be said that DNA damage do also occur in metabolic syndrome.

3.7: Correlation between NCEP/ATP III parameters and observed parameters with antioxidants

Present study contemplates the prediction as well as prevention of metabolic syndrome and DNA damage in diabetic animals after antioxidant supplementation. Though NCEP/ATP III criteria were involved herein to determine metabolic syndrome, other parameters analyzed throughout the study, were also taken into consideration. These parameters incorporated the analysis of serum PON-1 activity, time required for oxidation of sdLDL, concentration of lipid peroxides together with CRP, apo-B and TNF-α levels in serum, along with the estimation of DNA damage. In an effort to reveal a relationship between these factors and NCEP/ATP III parameters (FBG, TGs and HDL) in the diabetic rabbits with supplementation of vitamin C or E, Pearson's correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.3.7 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs and FBG, (ii) lipid peroxidation and HDL, (iii) sdLDL oxidation time and levels of TGs and FBG,

(iv) CRP and HDL, (v) apo-B and HDL, (vi) TNF-α and HDL, (vii) DNA damage and HDL. Thus it adds to an important observation depicting that the reduction in lipid peroxidation, and levels of CRP, apo-B and TNF-α together with the DNA damage occur due to an elevation in HDL concentration; an outcome of antioxidant supplementation. Similarly, the decrease in TGs and FBG concentration as a result of vitamin C or E supplementation was found to be responsible for increased PON-1 activity and the time required for oxidation of sdLDL. This implies that FBG, TGs and HDL levels play a fundamental role in regulation of several other metabolic syndrome parameters.

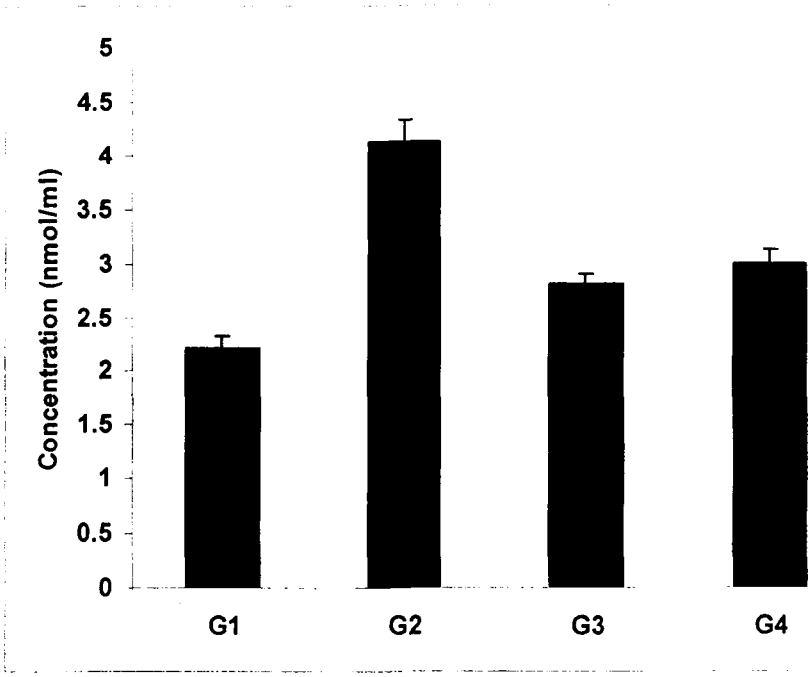


Fig.3.3: MDA levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in 'methods'. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

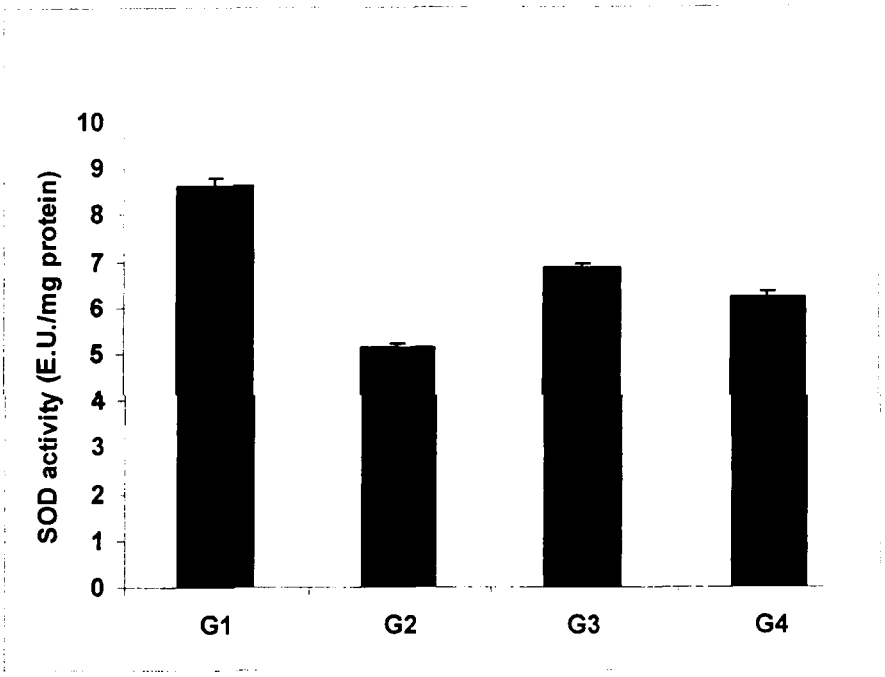


Fig.3.4(a): SOD activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The assay medium in a final volume of 3 ml consisted of 50 µl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit (E.U.) is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

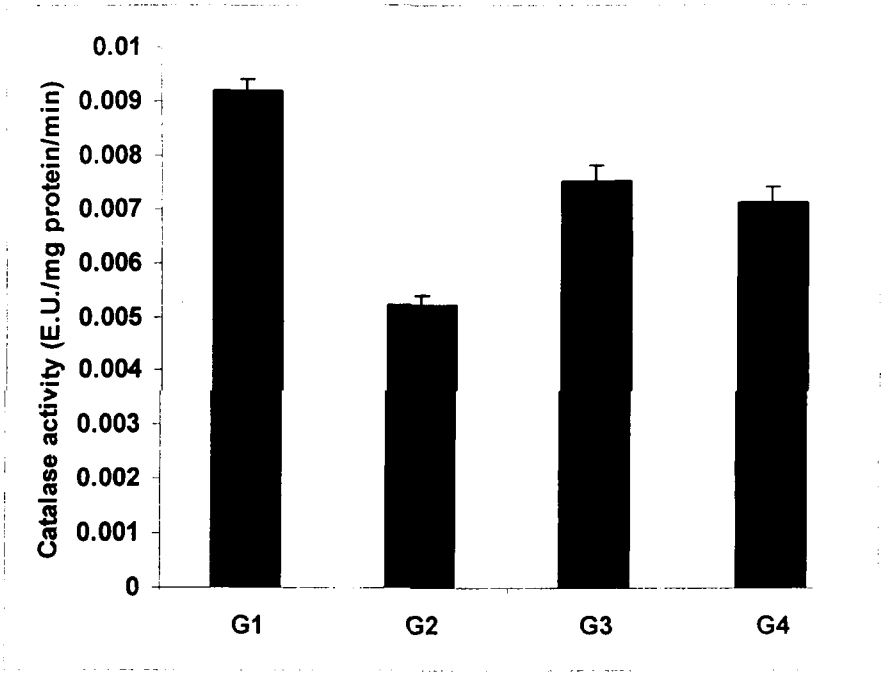


Fig.3.4(b): Catalase activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H_2O_2). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml H_2O_2 (30 mM) and 50 µl sample. One enzyme unit (E.U.) is defined as the amount of enzyme decomposing 1 µM H_2O_2 per min at 25°C. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

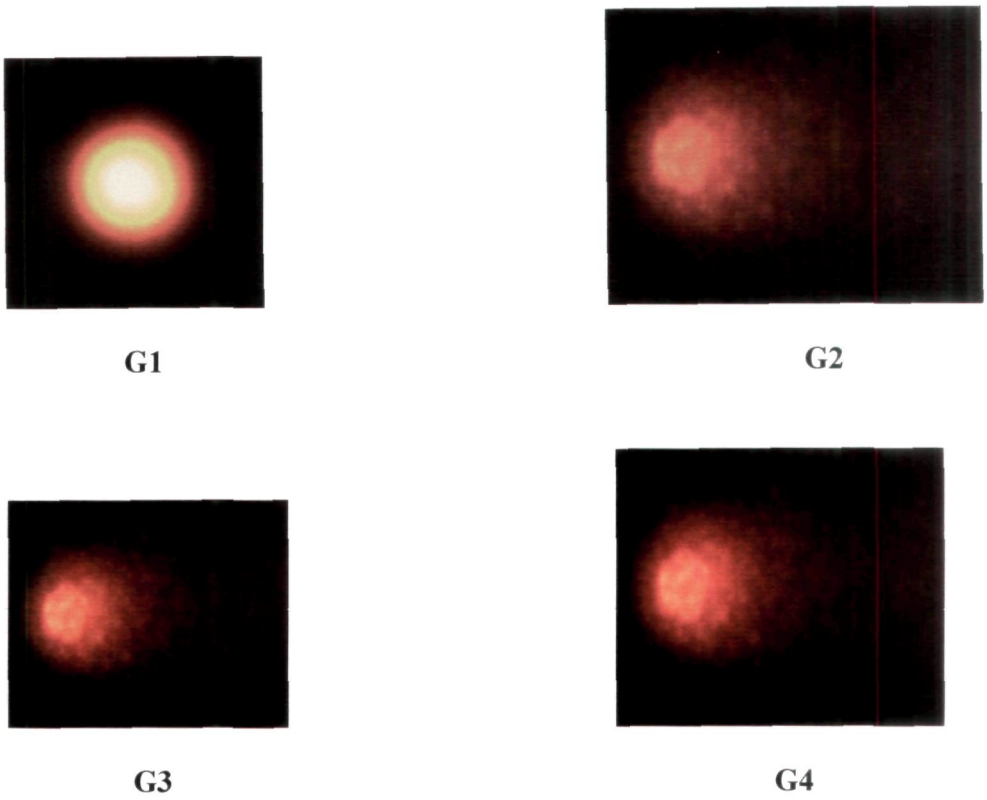


Fig.3.5(a): DNA damage in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants (representative photographs).

DNA breakage in the lymphocytes isolated from each rabbit, as measured by comet assay proposed by Singh et al. (1988) (details of the method are described in 'methods').

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

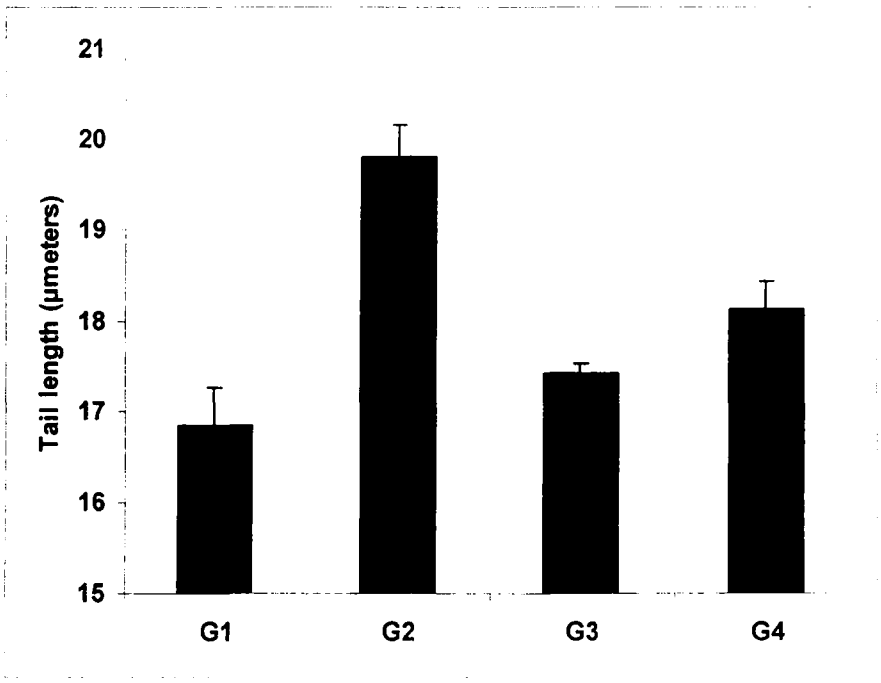


Fig.3.5(b): Extent of DNA damage in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

DNA breakage in the lymphocytes isolated from each rabbit was measured by comet assay proposed by Singh et al. (details of the method are described in 'methods'). Comet tail length (µmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

G1	Control
G2	Diabetic
G3	Diabetic + Vitamin C
G4	Diabetic + Vitamin E

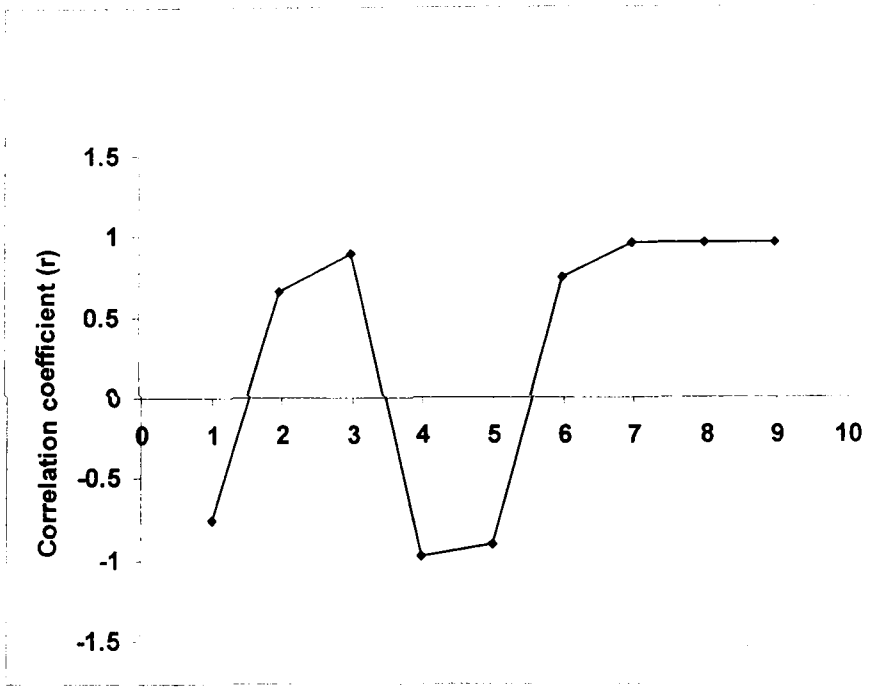


Fig.3.6: Correlation between DNA damage and parameters of metabolic syndrome as measured in diabetic rabbits.

Pearson's correlation analysis was performed to determine the relationship between DNA damage and the observed parameters of metabolic syndrome. The plotted values of correlation coefficient (r) indicate the positive or negative relationship between DNA damage and following parameters:

1 PON-1 activity
2 Lipid peroxidation
3 CRP concentration
4 sdLDL oxidation time
5 HDL concentration
6 TGs concentration
7 Fasting blood glucose levels
8 TNF-α concentration
9 Apo-B concentration

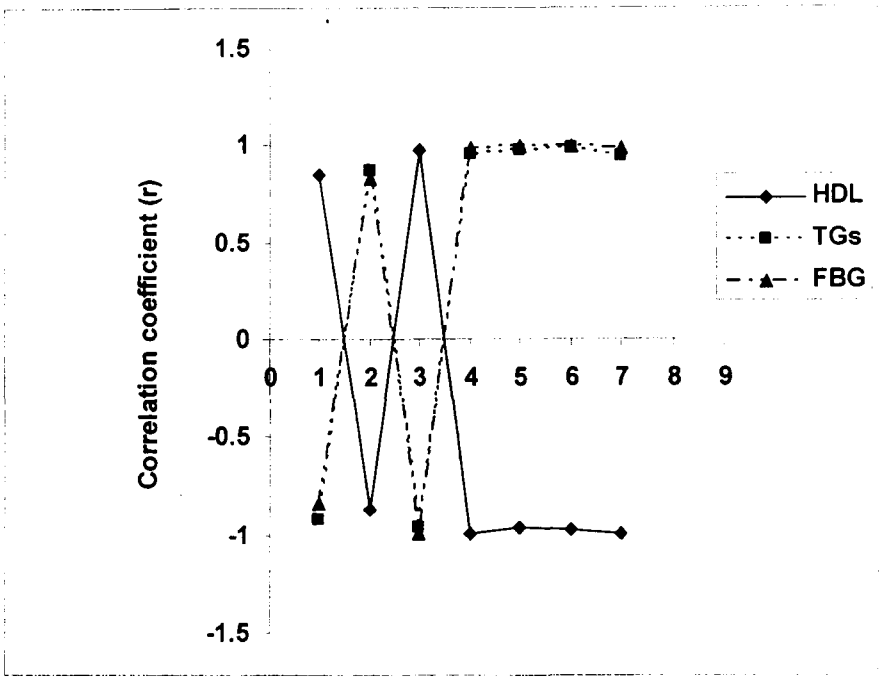


Fig.3.7: Correlation between NCEP/ATP III parameters and observed parameters with antioxidants.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed with vitamin supplementation. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1 PON-1 activity
2 Lipid peroxidation
3 sdLDL oxidation time
4 CRP concentration
5 Apo-B concentration
6 TNF-α concentration
7 DNA damage

Discussion

Metabolic syndrome, first described by Reaven in 1988, is characterized by a constellation of cardiovascular risk factors, including atherogenic dyslipidemia, abnormal glucose tolerance, hypertension, and visceral obesity, which are intimately associated with insulin resistance and hyperinsulinemia. Insulin resistance has been proposed to be the common denominator of the metabolic syndrome, but insulin resistance per se is not a compulsory element of the NCEP/ATP III definition used in the present study. Of the five criteria of metabolic syndrome defined in NCEP/ATP III, four (and notably hypertriglyceridemia, hypertension, hyperglycemia, and abdominal obesity) are independently characterized by the presence of systemic oxidative stress (Redon et al., 2003; Keaney et al., 2003). Furthermore, hypertriglyceridemia, hypertension, and obesity each is also associated with increased production of superoxide anion via the nicotinamide adenosine diphosphate oxidase pathway (Brasier et al., 2002). In addition, hyperglycemia leads to the formation of oxygen free radicals that may promote protein glycation and glucose autoxidation (Mezzetti et al., 2000).

Understanding the prevalence of a condition is helpful in defining the public health burden of that condition. Meaningful prevalence estimates are difficult to develop without a suitable definition however, which in the case of metabolic syndrome continues to be debated. The NCEP/ATP III definition is perhaps the most straightforward to implement because the five criteria are clearly defined. The present study therefore implied the same definition and its parameters to define metabolic syndrome in diabetic as well as hypertensive population. Given the high prevalence of metabolic syndrome and the associated risk of diabetes and CVD, an understanding of all facets of this syndrome is critical.

Earlier it has been established that increased plasma TGs and reduced HDL levels are the key features of metabolic syndrome. Although elevated LDL is not an integral characteristic, there is usually an increase in the

proportion of small, dense LDL particles. Together these abnormalities constitute the atherogenic dyslipidemia, as a component of metabolic syndrome (Krauss and Siri, 2004). Garin et al. (2005) have revealed significant, qualitative changes to lipoproteins in patients with this syndrome. Working on a similar ground, our results too have indicated qualitative changes in the lipid profile of diabetic and hypertensive subjects, thereby suggesting their risk for the development of metabolic syndrome. Moreover, the increase in waist circumference, FBG, as well as BP of the chosen population does satisfy the requirements of NCEP/ATP III to describe metabolic syndrome. These changes represent a net deterioration of the cardiovascular profile, which would contribute to an increased risk of coronary diseases and metabolic syndrome.

Oxidation of LDL is a process of lipid peroxidation in which the polyunsaturated fatty acids of LDL are successively degraded to different products (Brockes et al., 2003). The increased oxidative susceptibility of LDL together with the abnormal lipid profile like elevated TGs and low HDL levels, leads to increased CVD risk (Devaraj and Jialal, 1998). It has been observed that patients with hypertension have a significantly lower lipid peroxidation lag time as compared to normotensive subjects (Bracht et al., 1997). There is evidence of a genetic influence on the LDL subfraction pattern and possibly the atherogenic potential (Shimano et al., 1991). Previously it has been reported that the sub-fraction of LDL namely dense LDL, light LDL and very light LDL have different susceptibility for lipid peroxidation *in vitro* (Vasankari et al., 2001). The results of the present study therefore focus on the sub-fraction of LDL that is sdLDL as the literature points towards the sdLDL being particularly atherogenic (Vega and Grundy, 1996). Hypertensive patients have a preponderance of sdLDL particles, a phenomenon associated with atherogenic lipoprotein profile and a three-fold increased risk of CVD (Bracht et al., 1997). This may probably be due to the high susceptibility of sdLDL to oxidation. We have thus

demonstrated that the sdLDL of hypertensive subjects oxidized fastest as compared to that of diabetic and control subjects.

A surprisingly delayed oxidation of sdLDL in diabetic subjects may suggest that very high concentration of glucose could have actually acted as an antioxidant. This is in accordance with the report presented by Karabina et al. (2005) where incubation of HDL with glucose as high as 1M concentration, a quantity routinely used for generation of advanced glycation end products *in vitro*, lead to the preservation of the esterolytic activity of PON-1, an antioxidant enzyme associated with HDL. In contrast, when incubation of HDL was performed with physiologically normal (5 mM) or elevated glucose concentrations (up to 100 mM), there was a loss of esterolytic activity of PON-1. These data suggest that lower concentration of glucose may facilitate or sustain oxygen radical production whereas very high concentration might be self-quenching. There is precedence in literature for the "antioxidant" effects of glucose and other sugars (Chuyen, 1998), as molar concentration of mannitol has long been used as a radical scavenger (England et al., 1986). The delayed peak of oxidation in diabetes, as observed in our study, however is not suggestive of reduced risk of CVD, as the lipid profile of diabetic subjects remains abnormal with serum cholesterol, LDL and TGs elevated similar to hypertensive subjects. This could only be suggestive of elevated glucose acting as antioxidant and probably leading to delayed oxidation of sdLDL in diabetes as compared to hypertension.

HDL is known to potentially reduce oxidative modifications of LDL. The prevention of lipid peroxide formation during copper-induced LDL oxidation by HDL could be due to their enzyme content, such as PON-1. The exact mechanism by which PON-1 exerts its protective effect however, is not well established. It has been proposed that the antioxidant effect could be associated with the peroxidase-like activity of this enzyme. Thus, by hydrolyzing preformed lipid peroxides, PON-1 can delay the oxidation

induced by metal ions (Shih et al., 1998). Our results have shown a reduced PON-1 activity in hypertension as well as in diabetes being much reduced in hypertension. Thus, in hypertensive subjects, more qualitative changes occur to LDL which renders them more susceptible to oxidation; coupled to a reduction in the potential antioxidant activity of HDL. This is suggestive of hypertension being a higher risk for developing CVD compared to diabetes. Moreover, the TGs, LDL cholesterol as well as waist circumference in diabetic and hypertensive subjects remained elevated. Consequently, these are viewed as severe metabolic syndrome patients.

A relation between apo-B concentration and metabolic syndrome has previously been described in type 2 diabetic subjects (Relimpio et al., 2002). It provides a good surrogate measure of increased LDL particle numbers in people with metabolic syndrome and insulin resistance (Williams et al., 2003). The Framingham Heart Study established the best correlation between apo-B and small LDL particle number (Kathiresan et al., 2006). We have demonstrated that apo-B levels were increased in both diabetic and hypertensive subjects almost equally. This apo-B dysregulation may be caused by increased non-esterified fatty acid (NEFA) flux to the liver (Zhang et al., 2004) or by an altered cholesterol homeostasis (low cholesterol absorption and high cholesterol synthesis). The results discussed till now strongly suggest that along with qualitative, the quantitative aspects of cholesterol metabolism are equally important to assess the metabolic syndrome.

Recent cohort studies have demonstrated that CRP independently represents additive prognostic values at all levels of metabolic syndrome (Ridker et al., 2004). These studies also suggested a consideration of adding CRP as one of the clinical criterion for the assessment of metabolic syndrome. We have shown that CRP levels were significantly increased up to an approximately similar extent, in both diabetic and hypertensive subjects. This finding is consistent to recent reports suggesting the

association of high CRP levels with the presence of metabolic syndrome in population based studies (Frohlich et al., 2000; Festa et al., 2000). In a study of apparently healthy women to evaluate the relationship between CRP, metabolic syndrome, and incident CVD over an 8-year period of follow-up (Wu et al., 2002), CRP levels more than 3 mg/l at baseline added independent prognostic information of the metabolic syndrome at all levels of severity. Women with the metabolic syndrome showed a 4- and 2.6-fold increase in their risk for CVD depending on whether CRP levels were greater or less than 3 mg/l, when compared to those without the metabolic syndrome and having CRP levels lower than 3 mg/l. Similar augmented values of CRP were observed in the present study also where both diabetic as well as hypertensive population displayed CRP levels more than the control subjects; thereby suggesting them to be at a greater risk for the development of metabolic syndrome. It has been proposed earlier that in addition to being a marker of innate immunity, CRP also has several direct effects at the level of the vessel wall (Pasceri et al., 2001). Thus it is conceived that CRP is pro-atherogenic in endothelial cells. These observations along with basic research into the inflammatory mechanisms of both diabetes and vascular dysfunction (Pradhan and Ridker, 2002), provide strong evidence that insulin resistance and atherosclerosis share a common inflammatory basis.

The TNF-α gene locus seems to be involved in human insulin resistance-mediated hypertension (Pausova et al., 2000). Additional studies have suggested that TNF-α has an important role in the development of insulin resistance, both in obesity, and in NIDDM. When a soluble TNF-α-binding protein was infused into *fa/fa* rats, which have high levels of adipose tissue TNF-α, there was a 2-3 fold increase in insulin-stimulated glucose uptake, along with improved insulin receptor autophosphorylation in both adipose tissue and muscle (Hotamisligil et al., 1994). We have therefore monitored the changes in TNF-α levels in diabetic as well as

hypertensive subjects. As anticipated, the TNF-α levels of both the categories were found to be elevated. The hypertensive group however, showed comparatively greater elevation. Thus, suggesting an important role of TNF-α in developing metabolic syndrome, putting hypertensive population at a major risk. Consistent to our findings, Grundy (2003) has also suggested a significant association between inflammation, hypertension and metabolic syndrome. TNF-α is involved in the pathophysiology of hypertension in the metabolic syndrome and has also been known to stimulate the production of endothelin-1 and angiotensinogen (Kahaleh and Fan, 1997).

Hitherto, the measurement and observation of various components of metabolic syndrome in diabetic and hypertensive population has given an idea of the contribution of diabetes as well as hypertension to metabolic syndrome. In view of the requirement of an integrated approach for treatment of this cluster of diseases and its complications, medical treatments of traits of the metabolic syndrome should be scrutinized with respect to pleiotropic effects on hyperglycemia, hypertension, dyslipidemia, adipokines, and low-grade inflammation. Several studies have shown that treatments to improve insulin resistance or reduce low-grade sub-clinical inflammation also have beneficial effects on other components of the metabolic syndrome (Dagenais et al., 2001; Chiasson et al., 2003; Torgerson et al., 2004).

So far drug intervention studies in pre-diabetes have been performed only in people with impaired glucose tolerance (IGT). No data from controlled prospective studies are available for subjects with impaired fasting blood glucose (FBG). In subjects with IGT, about one-third is suffering from the metabolic syndrome. In the US Diabetes Prevention Program (DPP), metformin was compared with placebo and lifestyle modifications. It is remarkable that administration of metformin in diabetic subjects reduced the relative risk of developing metabolic syndrome by

21.7 % as compared to 14.4 % reduction attained by diet and exercise (Zimmet et al., 2003). Metformin is the only anti-diabetic drug that has been proven to reduce cardiovascular complications of diabetes, as shown in a large study of overweight patients with diabetes (UKPDS 34, 1998a). The International Diabetes Federation (IDF) has suggested the use of metformin in all cases inadequately controlled by non-pharmacological treatments (IDF, 2005). Metformin is therefore, said to be recommended by most guidelines as the drug of choice for monotherapy in diabetic patients.

Insulin is generally considered the most effective hypoglycemic treatment in type 2 diabetes (Nathan, 2007). In fact, unlike other medications, the therapeutic effect of insulin maintains a dose-response relationship in virtually any dose range; for this reason, it is conceivable that any degree of hyperglycemia can be corrected by insulin treatment, provided that adequate doses are administered. The UK Prospective Diabetes Study (UKPDS) have established that intensive therapy with insulin significantly reduced the diabetes related complications (UKPDS 33, 1998b).

An attempt was therefore made to observe the beneficial effects of metformin or insulin monotherapy against the diabetic complications leading to metabolic syndrome. It was manifested from our findings that in addition to an obvious reduction in the levels of FBG, TGs, and lipid peroxides, a significant increase in the serum PON-1 activity, sdLDL oxidation time as well as HDL levels was observed in the diabetic subjects receiving either metformin or insulin monotherapy. Nonetheless, the clear depiction of better control through metformin rather than insulin as observed in our study, is in agreement to the earlier reports validating the benefit to risk ratio for metformin was many fold greater than that for insulin in first line type 2 diabetes (UKPDS 34, 1998a).

Some researchers have indicated that metformin has anti-atherogenic effect (UKPDS 34, 1998a; Matsumoto et al., 2004). As for the mechanisms of

anti-atherogenic effects of metformin, recent studies have revealed that metformin significantly lowers insulin resistance and do possess anti-oxidant activity (Faure et al., 1999). A significant elevation in the serum PON-1 activity of diabetic subjects after metformin monotherapy, as observed in the present study, is also an indication of the antioxidant potential of metformin. Diabetic subjects treated with insulin monotherapy also showed comparable results however. Small-sized LDL particles are well known to be atherogenic (Austin et al., 1988) and normalization of LDL particle size is one of the probable anti-atherogenic changes. Induction of these changes by metformin or insulin administration may be one of the mechanisms of their anti-atherogenic effect. This cannot be directly suggested through our studies but there was a definite delay in sdLDL oxidation time after metformin as well as insulin monotherapy. Such a delay in oxidation could be due to either the lowering of sdLDL particle number or normalization of their size. Nonetheless, the quantification of sdLDL particles is necessary to confirm these findings.

An increase in TGs-rich lipoproteins which is one of the characteristics of dyslipidemia caused by insulin resistance (Ginsberg et al., 2005) is known to be related with the appearance of small-sized LDL (Reaven et al., 1993). In addition, the presence of increased small dense LDL particles may be related closely to the high predictive ability of cardiometabolic disorders and elevations in apo-B. It was also shown that high apo-B levels were more closely associated with central obesity, insulin resistance and inflammation (Sattar et al., 2004). Consistent to these findings, our results have also shown a significant decrease in the TGs and apo-B levels in diabetic subjects upon administration of metformin as well as insulin. A plausible explanation of these changes could be attributed to the reduction in hepatic gluconeogenesis, decreased absorption of glucose from the gastrointestinal tract, and increased insulin sensitivity upon metformin administration (Hundal et al., 2000). A recent study has shown

that metformin stimulates the hepatic enzyme AMP-activated protein kinase (AMPK), which plays an important role in the metabolism of fats and glucose (Zhou et al., 2001). AMPK stimulates both the catabolism of existing intracellular energy stores, such as TGs and an insulin-independent influx of extracellular energy sources, such as glucose (Hardie, 2003). On the other hand, the correction of abnormalities in the lipid profile after insulin administration may be the consequence of a balance maintained between lipid synthesis and breakdown, by insulin via acting as a strong anti-lipolytic agent (Howard et al., 1984). Previous studies have also reported a decreasing effect of metformin on very low-density lipoprotein (VLDL) (Hollenbeck et al., 1991), but the mechanism of this effect by metformin was not fully understood.

Increased inflammatory markers like TNF-α and CRP have been observed in patients with diabetes and hypertension, which are important pathobiological components of metabolic syndrome (Nishtha et al., 2008b). The measurement of these inflammatory markers thus provides a treatment target for metabolic syndrome. We observed minor decrement in the values of TNF-α and CRP upon metformin or insulin monotherapy in diabetic subjects. Earlier reports have also demonstrated no significant effect of glycemic control on markers of inflammation (Fonseca et al., 2006). Our findings consistently failed to demonstrate any significant anti-inflammatory properties of metformin or insulin. It is very well known that there exists a clear relationship between the number of metabolic disorders (dyslipidemia, obesity, insulin resistance, diabetes, and hypertension) and increasing CRP levels and the strongest association of high CRP levels have been observed with increasing central obesity and insulin resistance (Ford, 2003). Since our results with metformin or insulin monotherapy provided no changes at all in the increased waist circumference of diabetic subjects, a plausible explanation to unaltered inflammatory markers may be given thus.

Previous *in vitro* and *in vivo* studies have demonstrated that metformin causes an improvement in antioxidant activities in various tissues and also acts to limit lipid peroxidation (Kanigur-Sultuybek et al., 1995; Tessier et al., 1999; Srividhya and Anuradha, 2002). Analogous to these findings, our results have also demonstrated significantly reduced concentration of lipid peroxides in diabetic subjects after metformin as well as insulin monotherapy. The previously reported modification in the lipoprotein profile with similar treatment may be accountable for this important outcome. There is a good evidence for the involvement of enhanced oxidative stress in the pathogenesis of CVD and diabetes (West, 2000).

Free radicals can damage the double bonds of polyunsaturated fatty acids in the cell membrane, leading to a chain of chemical reactions called lipid peroxidation, during which aldehydes are formed. The measurement of malondialdehyde (MDA) by the thiobarbituric acid test is an indirect way of quantifying oxidative stress. We therefore, determined the levels of MDA in untreated diabetic subjects and those treated with metformin or insulin monotherapy. A significant elevation in the MDA levels of untreated diabetic subjects was noticed and hence perpetuates the relationship between diabetes and oxidative stress. On the other hand, decreased MDA levels were obtained with metformin as well as insulin monotherapy suggesting an important role of both of these hypoglycemics against oxidative stress. Similar outcomes with insulin monotherapy have been previously proposed by Konodo et al., (2002), where insulin therapy repressed LDL oxidation in diabetic subjects, which probably has resulted in decreased MDA levels. Therefore insulin therapy should also be acknowledged for its anti-oxidant potential; though in our study metformin showed better anti-oxidant property. It can thus be concluded that treatment with metformin may ameliorate the imbalance between free radical-induced increase in lipid peroxidation and decreased antioxidant

defences, by reducing the MDA level and increasing the enzymatic activities of SOD and catalase.

Bonnefont-Rousselot et al. (2003) also suggested that metformin could directly scavenge ROS or indirectly act by modulating the intracellular production of superoxide radicals. Thus, metformin may also help in protecting against free radical-induced-DNA damage. An opinion was therefore laid to verify the effect of metformin or insulin monotherapy in protection, if any, on the DNA damage incurred by free radical production during diabetes or metabolic syndrome. Therefore, we performed comet assay to investigate the DNA damage in peripheral blood lymphocytes obtained from diabetic subjects receiving either of the two monotherapies. Evidently, no significant changes were observed in the comet tail length obtained for diabetic as well as treated subjects. This result suggests that a suitable monotherapy with recommended doses of metformin or insulin in the newly detected diabetic subjects is unable to protect against DNA damage which may possibly be induced by oxidative stress.

These results strongly suggest that although the monotherapy with metformin or insulin succeeded in subsidizing a many of the components of metabolic syndrome, several issues are still unanswered. It was noticed that despite of beneficial changes in lipoprotein profile, such a monotherapy was unable to recompense for increased inflammatory markers and the DNA damage incurred in diabetic population. However, the association between various components of metabolic syndrome observed in this study and the parameters laid by NCEP/ATP III provided opportunity to understand the changes noticed before and after treatment in diabetic subjects. Thus correlation analysis serves as an important tool to justify the aberrations of these metabolic parameters using TGs and HDL. Out of the parameters analyzed in this study, those depicting elevated levels could be related directly to increased TGs and where the levels of parameters investigated were lower than normal, a direct relation with decreased HDL content can

be suggested. These findings are in agreement with the observations of Zak et al (2007); where positive correlation was found between the numbers of metabolic syndrome components and concentration of glucose and total cholesterol (TC), TGs, LDL, VLDL, and apo-B. The closest relation was found between the concentration of TGs and apo-B.

High blood pressure is a classical feature of the metabolic syndrome. It has been reported that the metabolic syndrome is present in up to one third of hypertensive patients (Schillaci et al., 2004). Blood pressure levels are strongly associated with visceral obesity and insulin resistance (Ferrannini et al., 1997), which are the main pathophysiologic features underlying the metabolic syndrome. It has been mentioned earlier that the components of metabolic syndrome are treated individually, there being no current treatment that can target all features. Some classes of antihypertensive drugs, notably calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists (ARAs), have been shown to reduce the occurrence of newly onset diabetes, particularly when compared with diuretics and ß-blockers (Sierra and Ruilope, 2003). It may therefore, be assumed that besides the lowering of blood pressure, certain antihypertensives may have different pleiotropic effects on the pathophysiology of the metabolic syndrome. ACE inhibitors and ARAs have also been shown to improve insulin resistance in many but not all studies (Kennedy et al., 2001). Furthermore, a reduction in excretion of albumin in the diabetic patients with microalbuminuria has also been observed upon administration of these drugs but their effect on lipid profile has been found to be insignificant (Sica and Bakris, 2002, Mann et al., 2003). In patients with the metabolic syndrome, ACE inhibitors or ARAs should therefore be the drugs of first choice.

In light of these observations, we chose ramipril and losartan, the well-known prototypes of ACE inhibitor and ARA, to verify whether they may or may not treat all the components of metabolic syndrome in

hypertensive subjects. Ramipril is one of the most frequently used ACE inhibitors in the treatment of hypertension in adults (Kaplan et al., 1993). Earlier studies on children with chronic kidney diseases associated with hypertension also put forward the efficacy of ramipril (Seeman et al., 2004). However, the newly introduced ARA (losartan) has been reported by some scientists to be more effective anti-hypertensive therapy because it leaves no option for regeneration of AT-II through non-ACE pathways (McConnaughcy et al., 1999).

Our results have demonstrated a significant elevation in PON-1 activity together with a reduction in lipid peroxidation as well as levels of inflammatory markers in hypertensive subjects treated with either ramipril or losartan as a once-daily anti-hypertensive agent. Surprisingly, the effectiveness of ramipril was found greater than that of losartan while analyzing each of the above reported parameters. ACE inhibitor ramipril has indisputably demonstrated reduced cardiovascular events in a broad range of high-risk patients who participated in the HOPE trial (Kennedy et al., 2001). Ramipril should therefore be a first choice antihypertensive agent in an individual with hypertension associated with metabolic syndrome. This may also be evident from the previous reports suggesting that ramipril can reduce cardiovascular morbidity and mortality by slowing the progression of atherosclerotic plaque formation, preserving endothelial function and reducing plaque activation independently of effects on blood pressure and lipid levels (Kennedy et al., 2001).

Visceral adipose tissue produces and secretes a number of adipocytokines, such as leptin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), angiotensinogen, and non-esterified fatty acids (NEFA), which induce hypertension (Katagiri et al., 2007). Thus abnormalities in inflammatory mediators have also been reported to be implicated with development of hypertension. A positive relationship between increased serum levels of CRP and the risk for development of hypertension was observed in participants of

the Women's Health Study (Sesso et al., 2003). In addition, serum TNF-α concentration has been reported to be positively correlated with systolic blood pressure and insulin resistance in humans (Zinman et al., 1999), and increased TNF-α secretion has been observed in monocytes from hypertensive patients (Dorffel et al., 1999). Hence, it is proposed that reduction of these inflammatory markers as a result of ramipril or losartan monotherapy in the hypertensive subjects could help to explore better prevention measures for metabolic syndrome.

After the seminal observations by Welborn et al (1966) and Modan et al (1985), an impressive amount of information was collected in support of the notion that essential hypertension is a condition characterized by multiple metabolic disturbances. It was observed that over-weight, IGT, hyperinsulinemia, low serum HDL, and high TGs levels were more frequently found in patients with essential hypertension than in normotensive subjects, regardless of age, gender, and ethnic background. As a result of epidemiologic analysis, and clinical investigations conducted by many research groups (Reaven, 1988; Ferrannini et al., 1991; Haffner et al., 1988), a blood pressure-centered point of view gradually has shifted in favor of a broader, more metabolic-oriented perspective. We therefore, also analyzed the variation, if any, occurred in serum HDL, TGs, and apo-B content of hypertensive subjects after ramipril or losartan monotherapy. The results obtained however, demonstrated only slight improvement. Correspondingly, no significant alteration was observed even in the oxidation time of sdLDL after any of the above drug treatments performed in hypertensive subjects. Consequently, the studies till this point suggest that though ACE inhibitors and ARAs have a significant role in lowering of blood pressure and inflammatory markers, the improvement of lipoprotein profile still remains a question. This is particularly important in patients with concomitant hypertension and dyslipidemia, because this group is at high risk for metabolic syndrome, and

in order to substantially reduce this risk, both BP and lipoprotein profile control must be achieved.

In rats metabolic syndrome could be induced by chronic consumption of high fat and high refined sugar (Barnard et al., 1998). Hypertension is therefore known to be associated with oxidative stress (Roberts et al., 2005), avid nitric oxide (NO) inactivation, and down-regulation of NO synthase (NOS) isoforms and endothelial NOS activator (Roberts et al., 2005). This suggests that oxidative stress and endothelial dysfunction may be strongly associated with development of hypertension in the metabolic syndrome. Recent evidences also propose that oxidative stress, which is elevated in the metabolic syndrome (Furukawa et al., 2004), is associated with sodium retention and salt sensitivity (Sarafidis and Bakris, 2007). It has also been suggested that formation of free radicals is increased in hypertensive subjects. Akin to these findings, our results have also demonstrated the involvement of oxidative stress in hypertension. The elevated MDA levels together with the decreased activity of antioxidant enzymes such as SOD and catalase in hypertensive subjects disturbs the critical balance between free radical generation and antioxidant defenses and hence causes oxidative stress. Surprisingly, the level of this oxidative stress marker was found to get reduced after ramipril or losartan monotherapy. A significant elevation in the activity of SOD and catalase was also observed in the hypertensive subjects being treated with these drugs. Nonetheless, much improved results were obtained with ramipril monotherapy suggesting it to possess better antioxidant potential compared to losartan. These results might provide an explanation for recovery of certain parameters following ramipril and losartan monotherapy.

Some studies have reported that DNA damage in subjects with hypertension was not significant as compared to the subjects without hypertension (Negishi et al., 2001). Our results, on the contrary, have shown an unambiguous and extended tail length of DNA belonging to hypertensive

subjects when compared to that of normotensive control subjects. Besides, it was also witnessed that neither ramipril nor losartan monotherapy could prevent the DNA damage up to any conspicuous level; as evidenced by almost similar tail-lengths of treated or non-treated subjects.

If one clinical axiom can be drawn from this available data, it is that the management of components of metabolic syndrome risk factors in isolation will not adequately reduce the risk of metabolic syndrome events. It therefore, greatly emphasized the need for development of some combination therapy or 'polypill' which may have a broad spectrum of actions for treating the low-grade inflammation, lipoprotein abnormalities and hence the complications associated with metabolic syndrome; keeping the role of "oxidative stress" as central.

Oxidative stress, an imbalance between prooxidant and antioxidant factors, in favor of prooxidants and thereby potentiating oxidative damage, may play a role in the pathophysiology of diabetes and CVD (Rao, 2002). Consequently, the question of whether antioxidants could have a beneficial effect on reducing the risk of these conditions, especially CVD, has been intensively investigated, but the results remain inconclusive (Asplund, 2002). If antioxidants play a protective role in the pathophysiology of diabetes and cardiovascular disease, understanding the physiological status of antioxidant concentrations among people at high risk for developing these conditions, such as people with the metabolic syndrome, is of interest.

The abnormal glucose metabolism in diabetes is also attributed to oxidative stress due to several factors. Hyperglycemia leads to the over-production of free radicals and the nonenzymatic glycation of proteins which exert deleterious effects on pancreas and liver. This hyperglycemia is accompanied with the increase in marked oxidative impact as evidenced by the significant increase in lipid peroxidation together with a decrease in the activity of antioxidant enzymes, such as SOD and catalase. Although quenching of free radical species, which is the principal mechanisms of action

of antioxidants, other mechanisms that affect the pathophysiology of diabetes and cardiovascular disease may be operating as well (Visioli, 2001). The effects of vitamins C and E have received a great deal of interest. Through effects on oxidation of LDL cholesterol, leukocyte adhesion, and endothelial function, vitamins C and E may slow atherosclerosis (Carr et al., 2000). These vitamins were also found to be positively associated with paraoxonase activity (Jarvik et al., 2002).

We have recently reported that the PON-1 activity together with oxidation time of sdLDL and concentration of HDL were significantly reduced while total TGs and CRP levels were elevated in metabolic syndrome patients (Nishtha et al., 2008b). It was also observed that the treatment of all of these anomalous parameters of metabolic syndrome was not possible by either the monotherapy for hyperglycemia using metformin or insulin or the monotherapy for hypertension using ramipril or losartan. It was therefore predicted that the best possible key to find a cure for metabolic syndrome could be the treatment of each of the associated complication or parameter. Our results with vitamin C and E demonstrated a significant improvement in each of the component of metabolic syndrome, being considered throughout the study.

Previous reports have suggested that increased DNA damage verified in the alloxan-induced diabetic animals was due to a significant disturbance in the antioxidant defense system (Damasceno et al., 2002). Our results have also indicated increased DNA damage in diabetic group, reinforcing the association of diabetes with oxidative stress. The damage was partially recovered when they were supplemented with vitamin C and the result obtained indicates that the protection could be acquired by maintaining the balance of excess free radicals and reduced antioxidant defenses. Vitamin E supplemented group however showed an insignificant reduction in the DNA damage. This is analogous to the earlier reports suggesting a rather

increased DNA damage after vitamin E supplementation in diabetic human subjects (Winterbone et al., 2007).

It has also been reported previously that both Vitamin C and E, the well-known scavengers of free radicals do facilitate the reduction in ROS production or help in promoting the antioxidant defense system (Parthiban et al., 1995). The amendment of abnormal metabolic syndrome parameters in antioxidant supplemented groups also implies the beneficial role of these vitamins against metabolic syndrome. Nevertheless, the extent of protection offered by vitamin C was higher than that with vitamin E. This may be attributed to the reason that vitamin E (tocopherol) is oxidized to tocopheroxyl radical upon reaction with a free radical and thus may itself act as a pro-oxidant sometimes (Bowry et al., 1995; Neuzil et al., 1997). Vitamin C on the other hand, serves as a co-antioxidant and regenerates tocopherol (Bowry et al., 1995; Packer, 1997) which is the active and reduced form of tocopheroxyl radical. This is a potentially important function because *in vitro* experiments have shown that tocopherol may lead to increased production of free radicals in the absence of vitamin C (Neuzil et al., 1997). However, the *in vivo* relevance of the interaction between vitamin C and vitamin E is unclear.

The precise mechanism for such a beneficial effect of these antioxidants, over the metabolic abnormalities that are directly linked to oxidative stress may be the scavenging of superoxide anions and its reactive oxygen intermediates (Ting et al., 1996). The concomitant decreased levels of MDA, as well as increased activities of SOD and catalase, rationally explain for the compensation in the complexities associated with metabolic syndrome upon antioxidant supplementation. It has long been postulated that supplementation with dietary antioxidants can alleviate the redox imbalance and thereby protect against the deteriorating effects of oxidative stress, progression of degenerative diseases, and aging (Pryor, 2000). Probably the anti-hyperglycemic or anti-hypertensive drugs do not possess

such specific ability of maintaining this redox imbalance and therefore are not sufficient enough to work against metabolic syndrome and its complications.

This is a novel study in the field of metabolic syndrome where the role of antioxidants has been identified against DNA damage and other related abnormalities. With the help of these evidences, it can now safely be said that DNA damage increases with diabetes and oxidative stress which is partially recovered by dietary supplementation of vitamin C. The correlation of DNA damage with the parameters of metabolic syndrome firmly suggests the occurrence of DNA damage in metabolic syndrome also. In light of these observations, it is plausibly suggested that vitamin C may be prescribed to the patients suffering from diabetes and metabolic syndrome in order to minimize the complications raised by oxidative stress. Human studies are nonetheless needed to confirm these findings.

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Small dense LDL oxidation in hypertensives and diabetics and prediction of Metabolic Syndrome

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KEYWORDS
Metabolic Syndrome;
Small dense LDL;
Oxidation;
Paraoxonase

Summary

Aim: The aim of this study is to compare the extent of small dense low-density lipoprotein (sdLDL) oxidation in diabetic and hypertensive patients and to investigate their correlation with Metabolic Syndrome as per NCEP ATP III criterion.
Design and methods: In the present study, 120 human subjects, men and women were selected randomly from the age group of 30–75 yrs for the screening of Metabolic Syndrome. Family history and prevalence of diabetes and hypertension in different age groups were recorded. Their waist circumference was measured and lipid profile was also determined. The sdLDL was isolated and oxidized in vitro. Attention was focused on the peak oxidation time of sdLDL in vitro and estimation of serum paraoxonase (PON-1) activity of all volunteers.
Results: Our results have indicated an overall increase in total cholesterol (TC), triglycerides (TGs), low-density lipoproteins (expressed as mg/dL) as well as mean waist circumference (expressed as cm) of the diabetic (242 ± 10.1, 218 ± 21.6, 140 ± 4.9, 105 ± 1.6) and hypertensive subjects (220 ± 6.4, 250 ± 12.9, 150 ± 5.6, 104 ± 1.9) as compared to controls (182 ± 5.4, 142 ± 8.9, 112 ± 4.3, 86 ± 1.5). It was also found that most of the diabetic and hypertensive subjects were from the age group of 50–59 yrs. The results obtained after oxidation of sdLDL have shown an early peak of oxidation in hypertensives [peak value = 65.3 ± 5.6 s] followed by diabetics [peak value = 75.7 ± 3.8 s]. Antioxidant enzyme paraoxonase was also found to be compromised in hypertensives and in diabetics.
Conclusion: The early peak of oxidation of sdLDL in hypertensives put them at a higher risk to CVD as compared to diabetics and abnormal lipid profile and increased waist circumference of diabetics and hypertensives suggest them to be potential patients of Metabolic Syndrome as per NCEP ATP III criterion.

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Introduction

The observation that certain risk factors accumulate in coronary patients is suggestive of a common origin and was the subject of quite early reports in clinical literature [1,2]. The concept has evolved through a number of synonyms [3] to its present status as the Metabolic Syndrome [4]. The strongest candidate for the common origin is insulin resistance [4,5]. A growing number of metabolic anomalies are linked to insulin resistance [3], the core components as excess weight, dyslipidemia and hypertension have recently been incorporated into guidelines by WHO [6] and National Cholesterol Education Programme Adult Treatment Panel III (NCEPATP III) [7] to provide a definition of the Syndrome which should aid clinical studies. Insulin resistance (or indications for abnormal glucose metabolism) is the central feature of WHO definition whereas the NCEP ATP III recommendations do not require evidence of insulin resistance.

Patients with the Metabolic Syndrome have significantly decreased low-density lipoprotein (LDL)–cholesterol/apo-A1 ratio which is related to the formation of small, dense lipoprotein particles that has higher susceptibility to oxidative modifications [8]. Hypertensive patients may have a preponderance of small dense LDL (sdLDL) particles; a phenomenon associated with an atherogenic lipoprotein profile and a 3-fold increased risk of cardiovascular diseases [9]. Additional risk factors that may be associated with the Syndrome include Type-II diabetes, hyperurecemia, microalbuminuria, and coagulation abnormalities that constitute a prothrombic diathesis [10]. Several studies have found an inverse relationship between the lag time of in vitro LDL oxidation and the severity and progression of coronary atherosclerosis [11], suggesting that enhanced susceptibility to oxidation may underlie the excess vascular disease observed in patients with diabetes.

The Syndrome is also associated with reduced concentrations and activities of the antioxidant enzyme paraoxonase-1 [8]. Human serum paraoxonase (PON-1), a 43 kDa protein catalyses the hydrolysis of organophosphate esters, aromatic carboxylic acid esters and carbamates in a calcium dependent manner [12]. Although the natural substrate for PON-1 is still unknown, several groups have reported that the enzyme protein has the capacity to retard the accumulation of lipid peroxides in low-density lipoprotein (LDL) and this is mainly due to the ability of the enzyme to reduce hydroperoxides [13]. Aviram et al. [14] have shown an inverse correlation between the esterolytic activity of PON-1 in serum and susceptibility of HDL to oxidation. The Metabolic Syndrome is well characterised by the presence of smaller, denser lipoprotein particles that increase their susceptibility to oxidative modification and a diminished serum PON-1 which is a major determinant of the antioxidant capacity of HDL. These may be contributory factors to the increased presence and severity of coronary diseases in such patients [8].

We through the present study, therefore have examined the kinetics of in vitro oxidation of sdLDL after isolating it from the serum of volunteers. Attention was focused on the peak obtained for oxidized sdLDL. The arylesterase activity of PON-1 was also recorded in the respective volunteers.

Subjects and methods

A total of 120 subjects were recruited on a consecutive basis for a screening program to check the prevalence of Metabolic Syndrome at the J.N. Medical College, A.M.U. Men and women aged 30–75 yrs participated in this study. All the volunteers, unaware of their health status and not observing any medication schedule for either diabetes or hypertension, were randomly selected and divided into three categories: diabetics (those having fasting plasma glucose level $\geq$110 mg/dL but no hypertension), hypertensives (those having BP $\geq$130/80 mmHg but no diabetes) and controls (those having neither diabetes nor hypertension). Demographic information and clinical history was obtained after getting informed consent. Waist circumference of all volunteers was measured using standard techniques. Fasting blood specimens were obtained for determination of plasma glucose, serum cholesterol, serum triglycerides (TGs), serum HDL and LDL cholesterol.

Plasma glucose was determined by the glucose oxidase peroxidase method [15] and the concentration of serum cholesterol, serum triglycerides, and HDL cholesterol were measured by an enzymatic colorimetric test using commercial kits from Ranbaxy Diagnostic Division, HP. LDL cholesterol was calculated using the Friedewald equation [16].

Small dense LDL-C assay

The details and validation of this experiment have been described elsewhere [17]. In brief, the precipitation reagent (0.1 mL) containing 150 U/mL of heparin sodium salt and 90 mmol/L $MgCl_2$ was added to a serum sample (0.1 mL) and the samples were incubated for 10 min at 37 °C after mixing. Then each sample was placed in an ice-bath and allowed to stand for 15 min after which the precipitates were collected by centrifugation at 15,000 rpm for 15 min at 4 °C. The precipitates were always

packed tightly at the bottom of the tube and the supernatant was clear. This heparin-Mg^{2+} supernatant contains sdLDL cholesterol with no influence of other lipoproteins [18]. Nitrogen gas was bubbled through the supernatant collected in separate tubes which are then closed tightly with parafilm and kept at 4 °C in dark to protect it from oxidative modification.

Oxidation of small dense LDL

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. [19] for LDL oxidation. 10 μL of supernatant containing sdLDL, collected above, oxygenated PBS and 32 μL of 1 mM $CuCl_2$ was added to a 2 mL quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored continuously at 37 °C on a UV spectrophotometer at 234 nm [20].

Measurement of arylesterase activity of PON-1 using phenylacetate as a substrate

Initial rates of hydrolysis were determined spectrophotometrically at 270 nm in a power wave 200 microtitre scanning spectrophotometer. The assays were performed in a final volume of 250 μL containing 1 mmol/L phenylacetate and 2 mmol/L $CaCl_2$ in 20 mmol/L Tris–HCl buffer, pH 8.0 in the presence of 0.1 μL of serum. The extinction coefficient at 270 nm for the reaction was 1307 mol/L/cm. One unit of arylesterase activity is equal to 1 μL of phenylacetate hydrolysed per milliliter per minute [21].

Statistical analysis

Analysis was performed using origin 6.1 statistical package for windows. Continuous variables were expressed as mean ± S.D. and qualitative data were expressed in percentages. Lipid profile, waist circumference, oxidation of sdLDL and arylesterase activity of PON-1 were compared by Student's *t*-test. *P*-value less than 0.05 were considered to be significant.

Results

A total of 120 subjects were included in the study and were divided into three categories, i.e. diabetics, hypertensives and controls (those having neither diabetes nor hypertension). Fig. 1 shows that among them 30% were diabetic, 13% were hypertensives and 57% were controls (as per their fasting plasma glucose and BP).

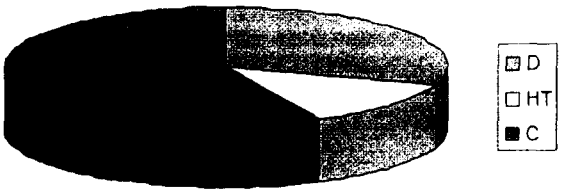

Figure 1 Prevalence of diabetes and hypertension among all the volunteers. Diabetics → volunteers whose blood glucose level ≥110 mg/dL. Hypertensives → volunvolunteers whose BP ≥ 130/85 mmHg. Controls → volunvolunteers having neither diabetes nor hypertension (as per their clinical history).

The information about waist circumference is an important parameter for the diagnosis of Metabolic Syndrome. Fig. 2(a and b) shows the mean waist circumference of men and women in all the three categories. It is clearly indicated that the mean waist circumference of diabetics (men = 105 ± 1.6, women = 95 ± 1.6) as well as hypertensives (men = 104 ± 1.9, women = 93 ± 2.4) was always higher than controls (men = 86 ± 1.5, women = 75 ± 1.6).

Fig. 3 gives the information about family history of all the study subjects. As shown in Fig. 3(a), 40% of diabetic subjects had positive family history and 60% had negative. Similarly, Fig. 3(b) indicates that among all the hypertensive subjects, 56% had the positive family history while 44% had negative.

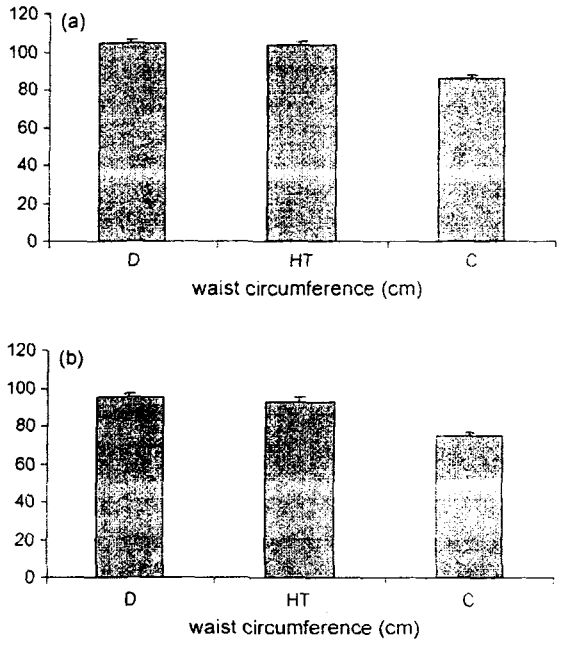

Figure 2 Mean waist circumference of diabetic, hypertensive and control subjects: (a) men and (b) women. Waist circumference ≥102 cm for men and ≥88 cm for women defines Metabolic Syndrome (NCEP ATP III guidelines). Results are mean ± S.E.M. $P < 0.05$, using paired *t*-test.

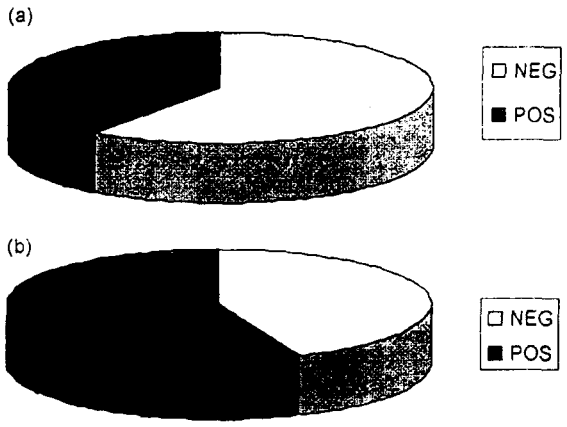

Figure 3 Family history of (a) diabetic and (b) hypertensive subjects. Results are expressed as *n*(%).

Fig. 4 shows the age related prevalence of diabetic and hypertensive subjects. It is indicated in both Fig. 4(a and b) that the prevalence of diabetes and hypertension increased with age and the maximum prevalence was seen in the age group of 50–59 yrs.

The lipid profile of diabetic, hypertensive and control subjects is shown by determining their total cholesterol (TC), TGs, HDL and LDL (Fig. 5(a–d)). The average cholesterol of diabetics (242 ± 10.1) was found to be maximum followed by hypertensives (220 ± 6.4) and controls (182 ± 5.4). Mean triglycerides were highest in hypertensives (250 ± 12.9), then in diabetics (218 ± 21.6) and lowest in controls (145 ± 8.9). HDL was almost stable in all cases (diabetics = 45 ± 1.1, hypertensives = 44.3 ± 1.4 and

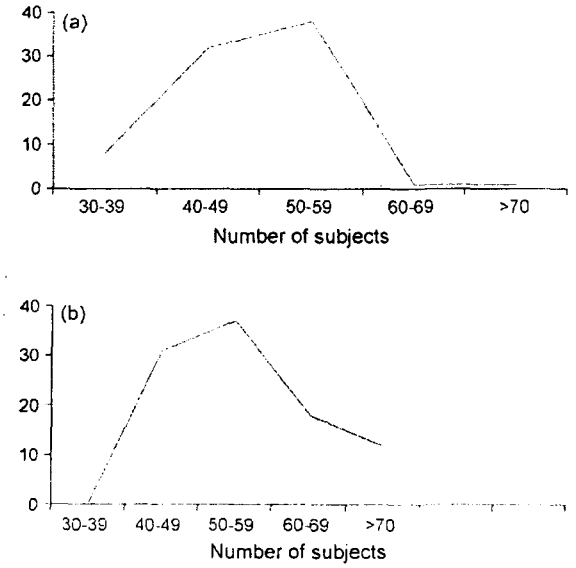

Figure 4 Age related prevalence of (a) diabetic and (b) hypertensive subjects. Results are mean ± S.E.M.

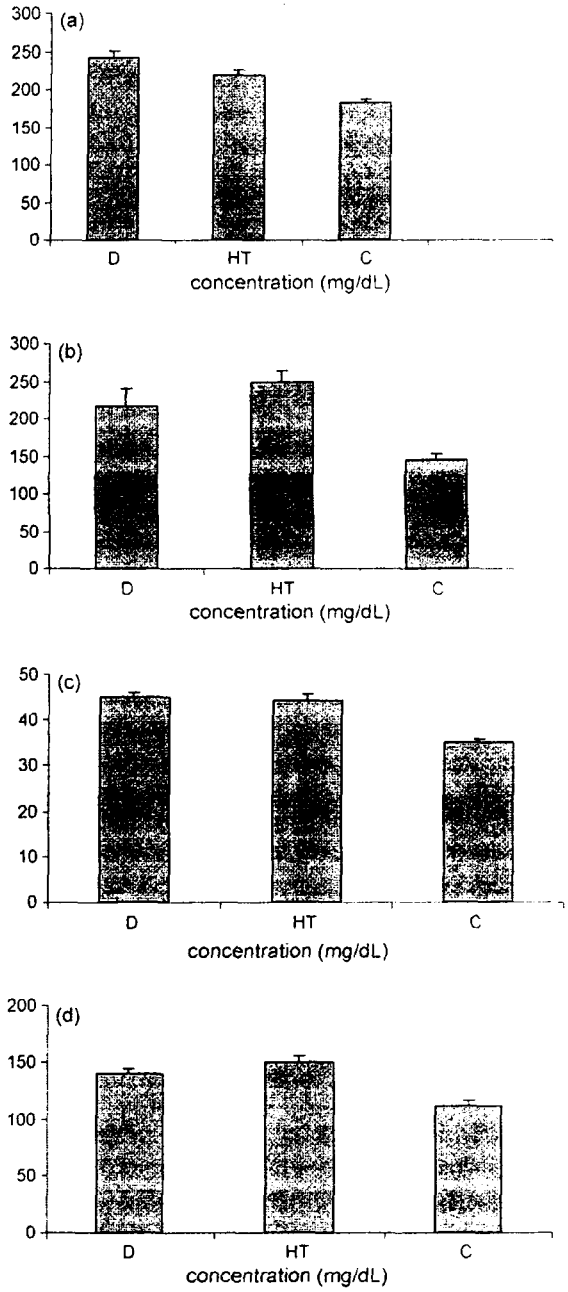

Figure 5 (a) Total cholesterol levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. $P < 0.05$, using paired *t*-test. (b) Triglycerides levels in diabetic, hypertensive and control subjects. TGs ≥150 mg/dL defines Metabolic Syndrome (NCEP ATP III guidelines). Results are mean ± S.E.M. $P < 0.05$, using paired *t*-test. (c) HDL levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. $P < 0.05$, using paired *t*-test. (d) LDL levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. $P < 0.05$, using paired *t*-test.

Table 1 Average peak value of oxidation of small dense LDL (sdLDL) of diabetic, hypertensive and control subjects

	Peak (s)
Hypertensives	65.3 ± 5.6
Diabetics	75.7 ± 3.8
Controls	86.5 ± 4.1

Results are mean ± S.E.M. Early peak of oxidation of sdLDL of hypertensives shows its greater susceptibility to oxidation in them followed by diabetics and controls.

controls = 35 ± 0.95) while LDL was highest in hypertensives (150 ± 5.6), followed by diabetics (140 ± 4.9) and controls (112 ± 4.3).

Recently sdLDL has been highlighted as a new risk factor for coronary heart diseases (CHD). It was discovered that the combination of Heparin and Magnesium precipitated a part of LDL which remained in the supernatant and was identified as sdLDL which was identical to the one isolated by ultracentrifugation. This supernatant was used as a source of sdLDL in the present study. It was oxidized in vitro by using $CuCl_2$ as described in Section 'Subjects and methods' [19] and the kinetics was observed in terms of peak time. Our results indicate the earliest peak of oxidation of sdLDL for hypertensive patients followed by diabetic and control subjects (Table 1).

The HDL-associated enzyme PON-1 has been identified as an important determinant of the capacity of HDL to prevent oxidative modification to LDL [14] and the activity of this enzyme has also been reported to be decreased in cardiovascular diseases (CVD) [22] and in diabetes [23]. Therefore we have also measured the arylesterase activity of PON-1 in all the three categories (Fig. 6). Our results have shown that the PON-1 activity was significantly reduced in case of hypertensives (0.26 ± 0.18) followed by diabetics (0.39 ± 0.14) and then by control subjects (0.60 ± 0.14).

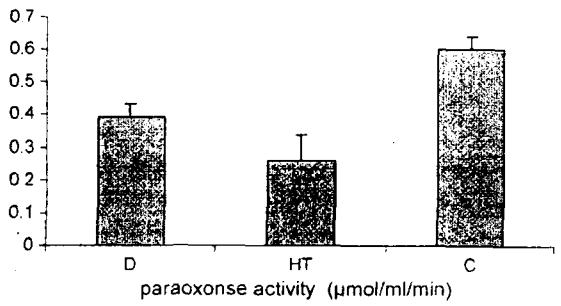

Figure 6 Arylesterase activity of PON-1. Results are mean ± S.E.M. $P < 0.05$, using paired t-test. The E_{270} for the reaction is 1307 mol/L/cm and 1 unit of arylesterase activity is equal to1 μmol of phenylacetate hydrolysed per milliliter per minute.

Discussion

The clustering of multiple metabolic abnormalities including obesity, insulin resistance, non-insulin dependent diabetes mellitus (NIDDM), hypertension and dyslipidemias have become known as the Insulin Resistance Syndrome or Multiple Metabolic Syndrome [24]. An elevation in blood pressure is one of the components of Metabolic Syndrome [25] and high blood pressure is a major risk factor for cardiovascular diseases [26]. Therefore, the increased risk of cardiovascular diseases in hypertensive patients correlates with blood pressure and may be related to other factors like abnormal lipid profile [27]. Studies have also shown an association between hypertension and the oxidation of LDL and particularly the fact that its susceptibility to oxidation is greater in patients with essential hypertension than in normotensive subjects [28,9]. It is suggested that oxidative modification of LDL could promote and accelerate the development of atherosclerosis [29,30]. When LDL is chemically modified, an uncontrolled uptake of oxidized LDL by the scavenger receptors in macrophages occur. As a consequence, they dedifferentiate into foam cells that accumulate in the arterial wall, forming early sclerotic lesions [31].

Oxidation of LDL is a process of lipid peroxidation. The polyunsaturated fatty acids of LDL are successively degraded to different products [32]. The oxidative susceptibility of LDL is increased when combined with cardiovascular risk factors like the abnormal lipid profile [33]. It has been observed that patient with hypertension has a significantly lowered lipid peroxidation lag time compared to normotensive subjects [9]. There is evidence of a genetic influence on the LDL sub-fraction pattern and possibly the atherogenic potential [34]. Previously it has been reported that the sub-fraction of LDL, namely dense LDL, light LDL and very light LDL have different susceptibility for lipid peroxidation in vitro [35] The results of the present study therefore focuses on the sub-fraction of LDL, that is sdLDL as the literature points towards the sdLDL being particularly atherogenic [36]. Hypertensive patients have a preponderance of sdLDL particles, a phenomenon associated with atherogenic lipoprotein profile and a three-fold increased risk of cardiovascular disease [9]. This may probably be due to the high susceptibility of sdLDL to oxidation. We have demonstrated that the peak time of oxidation of sdLDL was earliest in hypertensives as compared to controls.

Surprisingly, in diabetics the peak time was much delayed as compared to that in hypertensives. This probably was due to the fact that very high concentration of glucose in blood may act as an antioxidant [37] as there is precedence in literature for the "antioxidant" effects of glucose and other sugars [38] and molar concentration of mannitol has long been used as a radical scavenger [39]. Lower concentration of glucose may facilitate or sustain oxygen radical production whereas very high concentration might be related to the ability of glucose to generate advanced glycation end products (AGE), or to initiate oxygen radical production [40]. The delayed peak of oxidation in diabetes may not be suggestive of reduced risk of cardiovascular diseases as the lipid profile of diabetics remains abnormal with serum cholesterol, LDL and TGs elevated as in hypertensives. This could only be suggestive of elevated glucose acting as antioxidant and probably leading to formation of advanced glycation end products.

HDL have been shown to potentially reduce oxidative modifications of LDL. The prevention of lipoperoxide generation during copper-induced LDL oxidation by HDL could be due to their enzyme content, such as paraoxonase-1 (PON-1). The exact mechanism by which PON-1 exerts its protective effect is not well established. It has been proposed that the antioxidant effect could be associated with the peroxidase-like activity of this enzyme. Thus, by hydrolysing preformed lipid peroxides, PON-1 can delay or inhibit the initiation of oxidation induced by metal ions [41]. Our results have shown that the serum levels of the enzyme are reduced in patients with hypertension and diabetes being much reduced in hypertensives. Thus, in hypertensives, more qualitative changes occur to LDL which render them more susceptible to oxidation; coupled to a reduction in the potential antioxidant activity of HDL. This is suggestive of hypertensives as high-risk group for CVD as compared to diabetic patients. Moreover, the TGs, LDL cholesterol as well as waist circumference in diabetic and hypertensive subjects remained elevated. So these are viewed as a severe form of Metabolic Syndrome patients. Further studies are however needed to conclusively suggest the relation between oxidation of sdLDL and increased risk of CVD.

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